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Bovine Mastitis: Multidrug Resistance to Conventional Treatments and the Emerging Role of Antimicrobial Peptides Hs01 and Ds01 as Alternative Therapies

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Abstract: Bovine mastitis remains one of the most significant infectious diseases affecting dairy herds, generating substantial economic losses and continuous reliance on antibiotics. The indiscriminate use of conventional antimicrobials has accelerated the emergence of multidrug-resistant pathogens, reducing treatment success and threatening milk quality and food safety. This integrative literature review synthesized studies published between 2000 and 2025 from PubMed, Scopus, Web of Science, ScienceDirect, SciELO, and Brazilian databases, as well as reports from FAO, WOAH, and MAPA. Data on mastitis pathogens, resistance mechanisms, and alternative therapies were screened and analyzed descriptively. The literature reveals widespread resistance in *Escherichia coli* (Escherich, 1885) (Enterobacteriales: Enterobacteriaceae), *Streptococcus agalactiae* (Lehmann & Neumann, 1896) (Lactobacillales: Streptococcaceae), and *Staphylococcus aureus* Rosenbach, 1884 (Bacillales: Staphylococcaceae), and other major agents, emphasizing the limitations of standard antibiotic therapy and the urgent need for novel strategies. Among promising options, the synthetic antimicrobial peptides Hs02 and Ds01 demonstrate broad-spectrum activity, capacity to disrupt biofilms, and a low propensity to induce resistance. Their chemical synthesis allows structural modifications to improve selectivity and stability, offering a sustainable path to reduce antibiotic dependence and enhance mastitis control in dairy production.

Keywords: Antimicrobial, Antibiotic, Milk, Peptides, Production.

Research Paper

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1.0 INTRODUCTION

Mastitis is one of the oldest and most persistent diseases in dairy farming, with historical records dating back to early twentieth-century medical and veterinary descriptions, when the first control attempts involved rudimentary hygiene measures and the disposal of contaminated milk. With the progress of veterinary medicine over the decades, especially after the introduction of antibiotics in the 1940s, the disease underwent different management phases, ranging from empirical use of antimicrobials to the implementation of integrated control programs (FAO/WHO, 2021; Fonseca, 2021; Embrapa, 2023).

Since the 2000s, the accumulation of epidemiological data, the emergence of bacterial resistance, and the development of new diagnostic technologies have consolidated mastitis as one of the main focuses of animal-health research, driving public policies and global initiatives for milk-quality monitoring (Figure 1) (Ferrari *et al.*, 2024; Novartis, 2024; Sandnes, 2024).

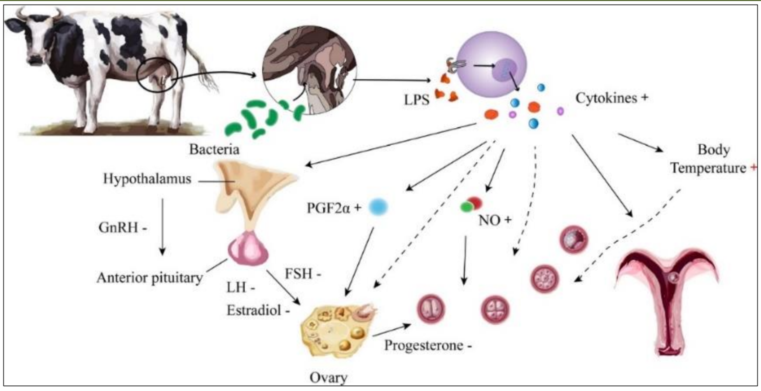

Figure 1: Mechanisms by which mastitis affects reproduction in dairy cows: A review of reproduction in domestic animals
Sources: Wiley Online Library, Wang *et al.* (2021), and Doi: 10.1111/rda.13953

1.1. Economic Impact of Mastitis

On a global scale, bovine mastitis is recognized as the disease with the greatest economic impact on milk production, affecting both developed and developing countries. In high-technology regions such as Europe and North America, prevalence is monitored by strict control programs. Yet, it still represents significant losses, with estimated costs reaching billions of dollars annually (Ferrari *et al.*, 2024; Novartis, 2024)

In developing countries, where the adoption of good milking practices and refrigeration infrastructure still faces challenges, incidence remains high, compromising the quality and safety of marketed milk. These data reinforce the need for integrated strategies that consider regional differences in management, climate, and herd genetics for effective disease control (NMC, 2020; WHO, 2023; Sandnes, 2024).

The economic impact of mastitis extends beyond decreased milk production. Direct costs include expenses such as medications, laboratory tests, veterinary fees, and milk disposal during the withdrawal period after treatment. Indirect costs involve reduced cow longevity, loss of quality bonuses, and the need for animal replacement. (Journal Campos Soberano, 2024; Novartis, 2024).

Brazilian studies show that each affected cow can cause annual losses that vary depending on infection severity and management effectiveness, with subclinical mastitis being the main unseen cause of losses in large herds. Programs like Normative Instruction No. 76/2018 and its updates aim to establish stricter standards for somatic cell and microorganism counts in milk, promoting the adoption of hygienic milking practices (Journal Campos Soberano, 2024; Novartis, 2024; Sandnes, 2024).

1.2. Etiological Agents, Infection Mechanisms, and Bovine Mastitis

The etiology of mastitis is multifactorial, involving contagious, environmental, and opportunistic microorganisms that find a favorable environment for colonization and multiplication in the mammary gland. Among the most common agents are *Escherichia coli* (Escherich, 1885) (Enterobacteriales: Enterobacteriaceae), *Klebsiella pneumoniae* (Schroeter 1886) Trevisan 1887 (Enterobacterales: Enterobacteriaceae), *Streptococcus agalactiae* (Lehmann & Neumann, 1896) (Lactobacillales: Streptococcaceae), *Staphylococcus aureus* Rosenbbach, 1884 (Bacillales: Staphylococcaceae), and *Streptococcus uberis* (Diernhofer, 1932) (Lactobacillales: Streptococcaceae) (Table 1) (Ferrari *et al.*, 2024; Novartis, 2024; Sandnes, 2024; Quintana-Castanedo *et al.*, 2025).

Table 1: Mastitis is mainly caused by *Escherichia coli* (Escherich, 1885) (Enterobacteriales: Enterobacteriaceae), *Klebsiella pneumoniae* (Schroeter, 1886) Trevisan 1887 (Enterobacterales: Enterobacteriaceae), *Streptococcus agalactiae* (Lehmann & Neumann, 1896) (Lactobacillales: Streptococcaceae), and *Staphylococcus aureus* Rosenbach, 1884 (Bacillales: Staphylococcaceae)

Etiological Agent	Predominant Type of Mastitis	Virulence Characteristics	Reported Antimicrobial Resistance
Staphylococcus aureus	Chronic / Subclinical	Biofilm formation, intracellular invasion	High, especially to beta-lactams
Escherichia coli	Acute clinical	Endotoxins, rapid multiplication	Variable: cefalosporin resistance in some herds
Streptococcus agalactiae	Subclinical contagious	Persistent colonization of the teat canal	Moderate, with cases of macrolide resistance

Etiological Agent	Predominant Type of Mastitis	Virulence Characteristics	Reported Antimicrobial Resistance
Streptococcus uberis	Environmental / Subclinical	Epithelial adhesion, enzyme production	Emerging resistance to tetracyclines
Klebsiella spp.	Severe clinical	Protective capsule, endotoxins	High in farms with frequent aminoglycoside use

These data emphasize that both contagious and environmental pathogens strongly influence mastitis in dairy cattle. *S aureus* and *S. agalactiae* are particularly relevant due to their persistence within the mammary gland and ability to spread between animals. In contrast, *E. coli* and *K. pneumoniae* are typically associated with acute clinical cases, often leading to severe outcomes. In addition, *S. uberis* represents a major challenge during the dry period, highlighting the multifactorial nature of the disease and the need for integrated prevention and treatment strategies (Barboza *et al.*, 2022; Bradley *et al.*, 2023; Embrapa, 2023; Okeke *et al.,* 2024; García-Alarcón *et al.,* 2025).

Bovine mastitis is associated with a wide range of microorganisms, whose identification is essential for both epidemiological understanding and selecting appropriate control strategies. Among the most common intramammary pathogens are *S. agalactiae*, *S. dysgalactiae*, and *S. uberis*. Environmental bacteria, such as *S. aureus, E. coli, Corynebacterium bovis* Bergey *et al.,* 1923 (Approved Lists 1980) (Mycobacteriales: Corynebacteriaceae), and opportunistic species like *Nocardia* Trevisan 1889 (Approved Lists 1980) (Mycobacteriales: Nocardiaceae) can also play a significant role, especially under poor hygienic conditions (Bradley, 2002; Leelahapongsathon *et al.,* 2016).

The distribution of mastitis is global, and the disease remains highly prevalent in all countries with intensive dairy farming. Beyond its direct impact on animal welfare, mastitis generates substantial economic losses due to milk yield reduction and increased veterinary costs. Certain pathogens, such as *E. coli*, may also pose zoonotic risks, reinforcing the importance of surveillance and strict hygiene measures (Halasa *et al.,* 2007; Gomes *et al.,* 2016).

The pathogenesis of mastitis may occur through ascending infections, where pathogens from the environment penetrate the teat canal, leading to inflammation and elevated somatic cell counts. Endogenous infections arise from bacteria normally colonizing the glandular tissue, which become pathogenic under stressful conditions. In systemic infections, hematogenous dissemination allows microorganisms such as *Mycoplasma* Nowak 1929 (Mycoplasmatales: Mycoplasmataceae) or *Leptospira* Noguchi 1917 non-Swainson 1840 non Boucot, Johnson & Staton 1964 (Leptospiral: Leptospiraceae) to reach the mammary gland (Table 2) (Fox, 2009; Ruegg, 2017).

Table 2: Etiological agents, infection mechanisms, transmission routes, and prophylaxis in bovine mastitis in São Paulo, Brazil Etiological Agents *Streptococcus agalactiae, S. uberis, and S. dysgalactiae Staphylococcus aureus*

Etiological Agents	Infection Mechanisms	Transmission Routes	Prophylaxis Measures
Streptococcus agalactiae, S. uberis, S. dysgalactiae	Endogenous or ascending infection of the mammary gland	Contaminated milking equipment, teat canal	Regular screening, treatment of carriers, and good milking hygiene
Staphylococcus aureus	Chronic intramammary infection	Direct cow-to-cow transmission, the milker's hands	Post-milking teat dipping, segregation of infected cows
Escherichia coli and *Klebsiella* spp.	Environmental ascending infection	Dirty bedding, fecal contamination	Clean housing, proper manure management, and pre-milking disinfection
Corynebacterium bovis	Colonization of the teat canal	Hands, fomites, milking liners	Routine teat disinfection, replacement of liners
Mycoplasma spp., and *Leptospira* spp.	Systemic (hematogenous) infection	Bloodstream dissemination	Control of systemic diseases, vaccination, and biosecurity
Nocardia spp.	Opportunistic infection under poor hygiene	Soil, contaminated environment	Strict asepsis, elimination of contaminated materials

The epidemiological chain involves reservoirs such as asymptomatic cows, subclinical carriers, and environmental niches. Transmission occurs mainly through contaminated milking equipment, the hands of milkers, fomites, and occasionally vectors. The primary entry route is the teat canal, although systemic dissemination may occur in specific cases. Predisposing factors include inadequate milking practices, poor teat sanitation, residual milk, and high-yield cows under stress (Rainard & Foucras, 2018; Ashraf & Imran, 2020).

Preventive measures target both reservoirs and transmission pathways. These include early detection and treatment of subclinical cases, segregation of affected animals, regular cleaning and disinfection of equipment, and the use of pre- and post-milking teat dipping. Improvements in herd management, environmental hygiene, and adherence to good milking practices remain the cornerstone for reducing mastitis incidence worldwide (Figure 2) (Krömker & Leimbach, 2017; Ruegg, 2022).

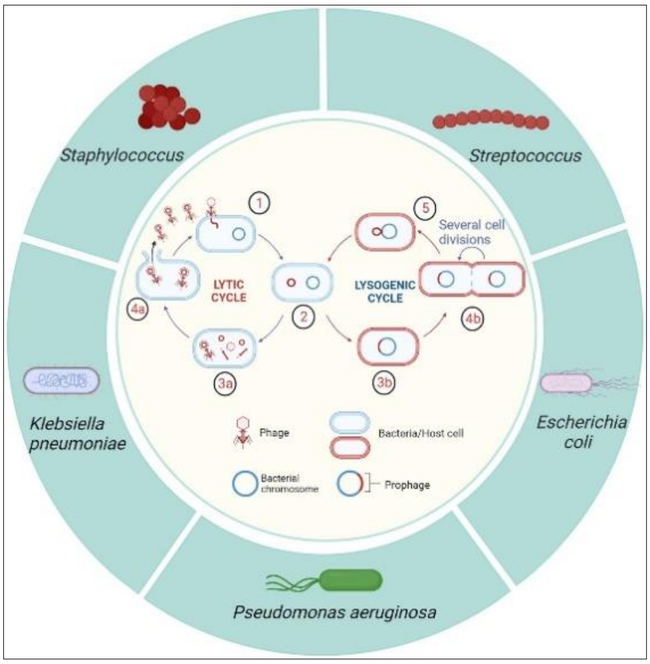

Figure 2: Mechanism of phage lysis of host bacteria and published types of mastitis pathogenic bacteria directed by phages. 1. The phage attaches to the host bacterium and injects DNA. 2. The phage DNA enters the lytic or lysogenic cycle. 3a. Synthesis of DNA and proteins followed by the assembly of new phages. 4a. Lyses the host bacterium, releasing large numbers of new phages. 3b. The phage DNA is integrated into the host bacterium's chromosome. 4b. Lysogenic bacteria reproduce normally. 5. Under specific conditions, the prophage is isolated from the host bacterium's genome and enters the lysis cycle
Source: BioRender.com

1.3. Formation of Biofilms and Antimicrobial Resistance

The formation of biofilms is one of the primary bacterial defense mechanisms against the host immune system and antimicrobial agents. Polysaccharide structures secreted by pathogens protect the colonies, hindering antibiotic penetration and the action of immune cells. This feature is particularly evident in *S. aureus*, whose intramammary persistence is directly associated with therapeutic failure and the need for culling chronically infected cows. Furthermore, the ability to internalize within epithelial cells allows the bacterium to temporarily escape immune responses, maintaining latent infection foci and predisposing to relapses even after prolonged treatments (Lima *et al.*, 2021; Embrapa, 2023; Novartis, 2024; PNH News, 2025; Quintana-Castanedo *et al.*, 2025).

Antimicrobial Resistance (AMR) has emerged as one of the greatest contemporary challenges in mastitis control. The indiscriminate or inappropriate use of antibiotics, combined with the absence of microbiological culture and sensitivity testing, favors the selection of multidrug-resistant strains. Recent studies report the presence of resistance genes to beta-lactams, macrolides, and tetracyclines in *S. aureus* and *E. coli* isolates from Brazilian and international dairy herds. This reality compromises the effectiveness of conventional treatments, increases the risk of residues in milk, and represents a public-health threat, as resistant microorganisms can be transmitted to consumers or the environment (MAPA, 2018a; FAO/WHO, 2021; Oliveira *et al.*, 2022; WHO, 2022).

AMR is a major challenge in the treatment of bovine mastitis, leading to frequent therapeutic failures even when recommended drugs are used correctly. Infections caused by *S. aureus* are particularly difficult to cure, and the persistence of resistant strains reduces the effectiveness of available antibiotics while posing a serious public health risk. The continuous use of antimicrobials in dairy herds is therefore recognized as an important driver of AMR (Figure 3) (Mera *et al.*, 2017; Karzis *et al.,* 2019; Leta *et al.,* 2020; Mphahlele *et al.,* 2020; García-Alarcón *et al.,* 2025).

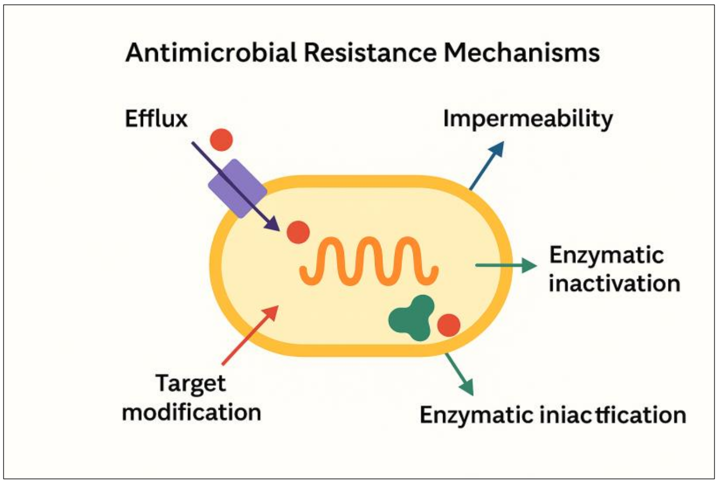

Figure 3: Antimicrobial resistance mechanisms. Bacterial structural diagram illustrating four mechanisms of antimicrobial resistance: efflux pumps, membrane impermeability, enzymatic inactivation, and target-site modification

Sources: Adapted from Bradley *et al.* (2023); WHO (2023)

Resistance arises naturally in bacterial populations but is accelerated by improper management practices. Factors that promote its development include indiscriminate or repeated antibiotic use, administration of subtherapeutic doses, incorrect treatment protocols, drug delivery by unqualified personnel, antimicrobial prescriptions without laboratory confirmation, and premature interruption of therapy. These conditions create selective pressure that favors resistant strains and compromises both animal health and milk quality (Figure 4) (Mera *et al.,* 2017; Karzis *et al.,* 2019; Leta *et al.,* 2020; Mphahlele *et al.,* 2020).

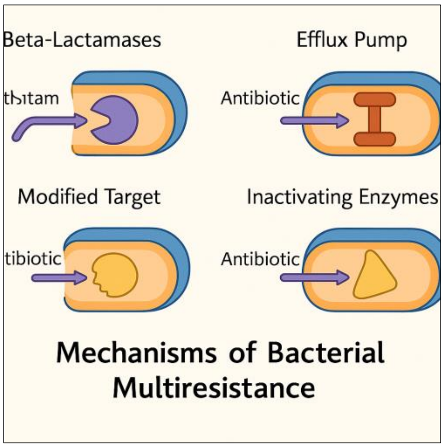

Figure 4: The main mechanisms of bacterial multiresistance, highlighting efflux pumps, membrane impermeability, enzymatic inactivation, and target-site modification that reduce antibiotic effectiveness in bovine mastitis pathogens

Sources: Adapted from WHO (2022); WOAH (2023)

The pathogenesis of mastitis involves the penetration of the infectious agent through the teat canal, adhesion to the mammary epithelium, bacterial multiplication, and activation of the local inflammatory response. Neutrophils and macrophages migrate to the infection site, releasing cytokines and reactive oxygen species that, while essential for defense, can damage mammary tissue and reduce the secretory capacity of epithelial cells (NMC, 2020; Lima *et al.*, 2021; Bradley *et al.*, 2023).

This inflammatory response is responsible for the increase in somatic cell count, one of the main laboratory indicators of subclinical mastitis. The severity of the clinical picture depends both on the virulence of the agent and on the animal's immune competence, being influenced by factors such as lactation stage, heat stress, nutrition, and herd genetics (NMC, 2020; Lima *et al.*, 2021; Bradley *et al.*, 2023).

1.4. Diagnosis and Mastitis Therapies

Accurate and rapid diagnosis of mastitis is essential to guide appropriate treatment, reduce the indiscriminate use of antibiotics, and minimize economic losses. Traditionally, methods such as the California Mastitis Test (CMT), Somatic Cell Count (SCC), and bacterial culture are used to identify clinical and subclinical cases. However, these procedures can be time-consuming or have sensitivity limitations. In recent years, advanced technologies such as real-time Polymerase Chain Reaction (PCR), mass spectrometry, and direct bacterial DNA detection tests in milk have enabled faster and more specific results, facilitating the implementation of targeted therapies at the onset of infection (Figure 5) (MAPA, 2018a; Oliveira *et al.*, 2022; Bradley *et al.*, 2023).

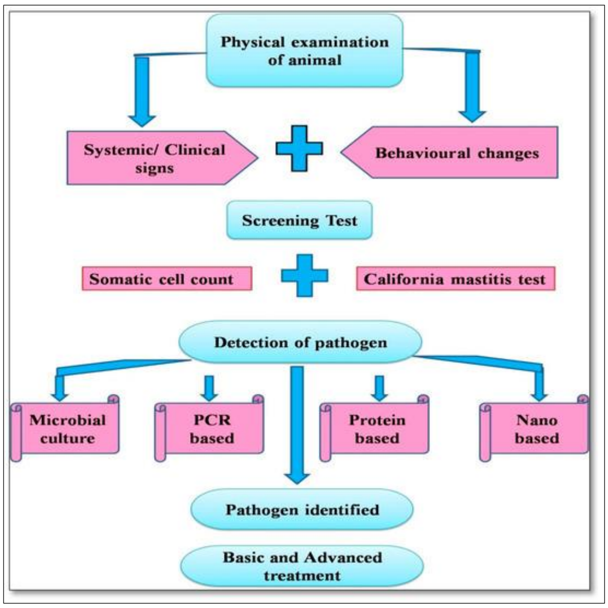

Figure 5: Overview of diagnoses of bovine mastitis. Treatment, reducing the indiscriminate use of antibiotics, and minimizing economic losses

Source: Doi: 10.3390/vetsci10070449

Electrical-conductivity sensors, temperature analyzers, and artificial-intelligence-based screening devices have been incorporated into robotic milking systems, enabling continuous monitoring of mammary-gland health. These resources detect subtle changes in milk and send real-time alerts, allowing the identification of cows in the early stages of infection before clinical signs appear. Automation of the milking process also helps reduce cross-contamination among animals and improves herd welfare by lowering stress during lactation (NMC, 2020; FAO/WHO, 2021; Lima *et al.*, 2021).

1.5. Therapeutic Field, in Addition to Conventional Intramammary Antibiotics and Antimicrobial Peptides

In the therapeutic field, in addition to conventional intramammary antibiotics, recent research has explored innovative alternatives for controlling mastitis. Specific bacteriophages have shown the ability to lyse multidrug-resistant strains of *S. aureus* and *E. coli*, offering a highly selective approach with a low risk of resistance development. Immunomodulatory therapies, such as next-generation vaccines and adjuvants capable of stimulating the innate immunity of the mammary gland, have been tested in experimental herds, showing promising results in reducing disease incidence and the severity of clinical cases (Bradley *et al.*, 2023; Embrapa, 2023; WHO, 2023; Novartis, 2025).

In recent years, research into bovine mastitis has focused on innovative bioactive molecules, such as antimicrobial peptides, which stand out for their ability to selectively act on the bacterial membrane. Unlike conventional antibiotics, peptides are short chains of amino acids capable of interacting with phospholipids in the cell membrane, destabilizing them and leading to the death of the pathogen. This mechanism reduces the likelihood of developing resistance, since membrane integrity is vital for bacterial survival (Nunes, 2023; Mota *et al.*, 2024; Zhao *et al.,* 2025).

Within this group, the experimental peptides Hs02 and Ds01 are noteworthy for their bactericidal activity against multidrug-resistant strains of *S. aureus* and *E. coli*, the most important microorganisms in bovine mastitis. In vitro assays demonstrate that these molecules can disrupt biofilms, penetrate the extracellular matrix, and reduce bacterial counts within hours, paving the way for shorter treatments with a lower risk of residues in milk (Figure 6) (Nunes, 2023; Mota *et al.*, 2024; Zhao *et al.,* 2025).

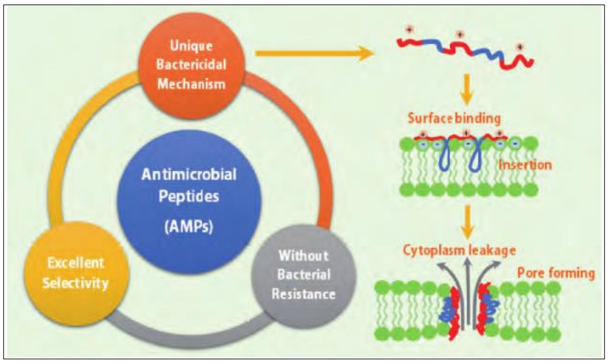

Figure 6: Antimicrobial peptides are short, positively charged molecules with broad antibacterial action and low risk of resistance. They damage microbial membranes and show selective toxicity, making them promising next-generation antibiotics. Because natural extraction is costly and slow, chemical synthesis enables large-scale production and custom design

Source: https://www.omizzur.com/knowledge/design-and-synthesis-of-antimicrobial-peptides.html

Synthetic antimicrobial peptides, including molecules like Hs02 and Ds01, stand out among emerging therapies. Initial studies show that these compounds have broad-spectrum bactericidal activity, can disrupt biofilms, and have a low tendency for resistance development. Although still in a pre-commercial phase, these molecules offer a promising option to replace or complement traditional antibiotics, especially in cases of mastitis caused by multidrug-resistant pathogens. Using these peptides may reduce the reliance on critically important antimicrobials and, in turn, minimize the risk of residues in milk intended for human consumption (Embrapa, 2023; Ferrari *et al.,* 2024; Novartis, 2024; Novartis, 2025).

Membrane-active Peptides (MAPs) offer promising antimicrobial applications but often lack selective action on bacterial membranes. Among them, Hs01, a 16-residue cationic intragenic peptide, exhibits broad antimicrobial and anti-inflammatory activity, along with moderate cytotoxicity. To enhance its therapeutic profile, two generations of shortened or point-mutated analogs were synthesized and evaluated by circular dichroism, antimicrobial assays, and BV-2 cell tests; second-generation variants, especially analog 16.3, achieved higher therapeutic indices against Gram-negative bacteria while activity against Gram-positive species declined, reflecting an optimized balance between cationic charge and hydrophobicity that preserved antimicrobial potency with reduced cytotoxicity (Figure 7) (Nunes, 2023; Mota *et al.*, 2024).

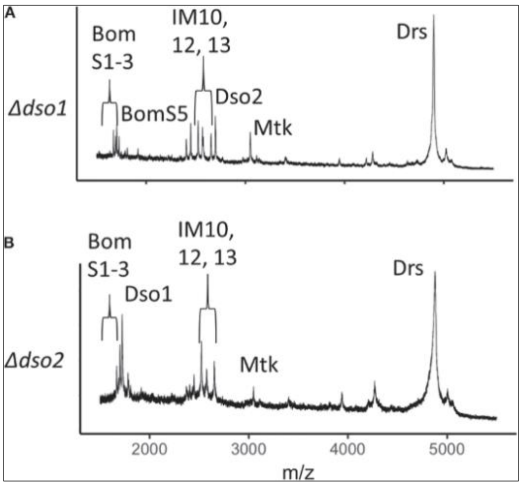

Figure 7: MALDI-TOF spectra for Ds01 and Ds02 hemolymph. (A, B) Mass spectrometry analysis of Toll-induced hemolymph in linear mode, highlighting loss of DS01 (A) and Ds02 (B) in deletion mutants

Source: https://www.researchgate.net/figure/MALDI-TOF-spectra-for-DDS01-and-Ddso2-hemolymph-A-B-Mass-spectrometry-analysis-of_fig4_338767558

Beyond its antimicrobial effects, Hs01 also demonstrated antineoplastic activity by reducing the viability of multiple leukemia cell lines without harming peripheral blood mononuclear cells, arresting Human Leukemia Cell Line (HL-60) cells in the Cell Cycle (G1 phase-mitosis), inducing membrane pore formation, and Lactate Dehydrogenase (LDH)) release, and up-regulating pyroptosis markers such as Inflammasome Sensor In Human Bronchial Epithelial Cells (NLRP1), Caspase-1 (CASP-1), Gasdermin E (GSDME), and Interleukin 1 beta (IL-1β), highlighting its potential as a template for future antimicrobial and anticancer peptide design (Figure 8) (Nunes, 2023; Mota *et al.*, 2024).

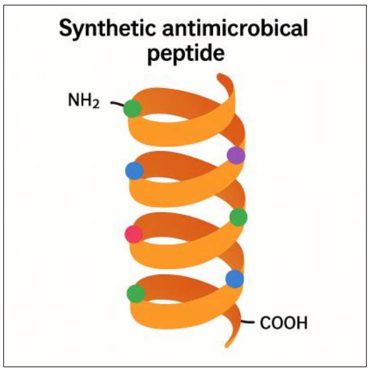

Figure 8: Synthetic antimicrobial peptide structure (Hs01/Ds01). Three-dimensional alpha-helical representation of a synthetic antimicrobial peptide, indicating the Amino (NH_2) and Carboxyl (COOH) termini
Sources: Adapted from Novartis (2024, 2025); Quintana-Castanedo *et al.* (2025)

1.6. Preventive Measures and Management Practices

The prevention of mastitis is based on a set of integrated measures involving strict hygiene, proper herd management, continuous monitoring, and worker education. The adoption of good milking practices is considered the first line of defense against the disease. Before milking, it is essential to disinfect the teats with appropriate solutions, such as iodine or chlorhexidine, followed by drying with disposable paper towels for each cow. This simple routine drastically reduces the microbial load on the teat surface and prevents the entry of pathogens during milking (MAPA, 2018a; FAO/WHO, 2021; Barboza *et al.*, 2022; Embrapa, 2023).

Post-dipping, which consists of immersing the teats in disinfectant solutions immediately after milking, is another fundamental pillar of prevention. This practice eliminates microorganisms that may have adhered to the skin during milk removal and protects the teat canal while the sphincter remains open, a period when the gland is more vulnerable to bacterial penetration. Studies show that farms correctly applying post-dipping experience a significant reduction in the incidence of both clinical and subclinical mastitis, in addition to lower somatic cell counts in bulk milk tank(Table 3) (NMC, 2020; Lima *et al.*, 2021; Bradley *et al.*, 2023; WHO, 2023; Ferrari *et al.*, 2024).

Table 3: Effective practices include pre- and post-dipping, dry-cow therapy, equipment maintenance, and clean bedding. These measures reduce new infections by 50–80% and lower somatic cell counts. Consistent hygiene and regular equipment calibration are essential to maintain milk quality

Preventive Measure	Evidence of Effectiveness	Practical Observations
Pre-dipping (pre-milking disinfection)	>70% reduction in new clinical cases	Use iodine or chlorhexidine solution
Post-dipping (post-milking disinfection)	Up to 80% reduction in S. aureus infections	Essential to protect the teat sphincter
Dry-cow therapy	50–80% reduction in infections in the next lactation	Combine a long-acting antibiotic and an internal sealant
Equipment maintenance	Significant reduction in environmental mastitis	Regular calibration and periodic liner replacement
Bedding and environment management	Marked reduction in E. coli mastitis	Frequent bedding replacement and adequate ventilation

Dry-cow therapy is a preventive approach aimed at eliminating existing subclinical infections at the end of lactation and protecting the gland during the resting period. It involves intramammary infusion of long-acting antibiotics or internal sealants immediately after the final milking, creating a physical and chemical barrier against pathogens. This practice has shown high effectiveness in reducing mastitis in the subsequent lactation and is recommended by milk-quality programs worldwide (MAPA, 2018b; NMC, 2020; Barboza *et al*., 2022; Embrapa, 2023; WHO, 2023; Ferrari *et al*., 2024).

Proper maintenance and sanitation of milking equipment are equally critical. Teat-cup liners, collectors, and vacuum lines must be cleaned and disinfected daily to prevent bacterial biofilm formation. Air leaks, improper pressure, and vacuum failures can cause lesions in the teat sphincter, favoring microorganism entry. Regular audits and calibration of milking systems are recommended to ensure that technical parameters remain within ideal limits, minimizing infection risks (Lima *et al.*, 2021; WOAH, 2022; WHO, 2023; Senar, 2024).

Nutritional management also plays a key role in mastitis prevention. Balanced diets in energy, protein, minerals, and vitamins strengthen the cows' immune response, reducing susceptibility to intramammary infections. Trace minerals such as selenium, zinc, and copper, as well as vitamins A and E, participate in antioxidant and immunomodulatory processes that enhance the efficiency of neutrophils and macrophages. Strategic supplementation programs, particularly during the pre-partum and early lactation periods, help maintain the integrity of mammary tissue and the natural resistance of the gland (FAO/WHO, 2021; Barboza *et al.*, 2022; Bradley *et al.*, 2023; Ferrari *et al.*, 2024).

Housing conditions must be kept clean, dry, and well-ventilated to avoid the proliferation of environmental pathogens such as *E. coli* and *Klebsiella* spp. Dirty and moist bedding exponentially increases infection pressure, especially in intensive production systems. Frequent bedding replacement, the use of absorbent materials, and proper manure management are measures that reduce teat exposure to mastitis-causing agents (Table 3) (Lima *et al.*, 2021; Embrapa, 2023; Ferrari *et al.*, 2024).

1.7. Brazilian and International Normative Instructions

Continuous training and education of milkers are essential for the success of preventive measures. Milking routines should be standardized, with clear procedures for pre-dipping, stimulation, milk removal, and post-dipping. Early detection of changes in milk, such as clots or discoloration, depends on the careful observation of workers. Educational programs addressing hygiene, biosecurity, and animal welfare increase adherence to recommended practices and contribute to the sustainable reduction of mastitis in the herd (Table 4) (NMC, 2020; WOAH, 2022; Ferrari *et al.*, 2024; Senar, 2024).

Table 4: Brazilian and international guidelines MAPA Normative Instructions 76/2018 and 77/2018, NMC, EU Directive 92/46/EEC establish strict limits for SCC and TBC and require hygienic milking and self-control programs. State quality-payment plans and manuals from Embrapa and FAO/WHO promote good agricultural practices to reduce antibiotic use. Continuous training (SENAR) supports prevention, early detection, and rational antimicrobial application

Regulation/Protocol	Main Content	Organization/Year
Normative Instruction No. 76/2018 (MAPA)	Establishes criteria for raw refrigerated milk quality, setting limits for Somatic Cell Count (SCC) and Total Bacterial Count (TBC).	Ministry of Agriculture, Livestock, and Food Supply / 2018
Normative Instruction No. 77/2018 (MAPA)	Defines requirements for milk collection and transport, mandating self-control programs by producers.	MAPA / 2018
State quality-payment programs	Financial bonuses for producers who maintain SCC and TBC within ideal ranges, encouraging good agricultural and milking practices.	State agencies and dairy industries – ongoing
National Mastitis Council (NMC) Guidelines	Protocols for hygienic milking, microbiological culture, rational antibiotic use, and the California Mastitis Test (CMT).	NMC, USA – continuous updates
Directive 92/46/EEC	Sets strict SCC and TBC limits and hygiene criteria for raw milk in the European Union.	European Union – 1992 and updates
Good Agricultural Practices Manual	Management, hygiene, nutrition, and environmental control recommendations to reduce antimicrobial use.	Embrapa – recent editions
Good Milking Practices Manual	International guidelines for hygiene, early detection of subclinical mastitis, and proper disposal of contaminated milk.	FAO/WHO – recent editions
SENAR Training Courses and Protocols	Training in mastitis prevention, diagnosis, and treatment with periodic updates.	National Rural Learning Service – ongoing

Nomenclature:

Ministry of Agriculture, Livestock, and Supply (MAPA), Somatic Cell Count (SCC), Thermal Barrier Coatings (TBC), Brazilian Agricultural Research Corporation (Emprapa), Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), and National Rural Learning Service (SENAR).

Continuous training and education of milkers are essential for the success of preventive measures. Milking routines should be standardized, with clear procedures for pre-dipping, stimulation, milk removal, and post-dipping. Early detection of changes in milk, such as clots or discoloration, depends on the careful observation of workers. Educational programs addressing hygiene, biosecurity, and animal welfare increase adherence to recommended practices and contribute to the sustainable reduction of mastitis in the herd (NMC, 2020; WOAH, 2022; Ferrari *et al.*, 2024; Senar, 2024).

The consolidation of mastitis control programs at national and international levels is essential to standardize prevention, diagnosis, and treatment practices, ensuring milk quality and food safety. In Brazil, Normative Instruction No. 76/2018 and Normative Instruction No. 77/2018 of the Ministry of Agriculture, Livestock, and Food Supply (MAPA) establish limits for Somatic Cell Count (SCC) and Total Bacterial Count (TBC) in raw refrigerated milk, as well as requirements for product transport and storage. This regulation encourages producers to continuously monitor herd health and adopt strict hygiene routines (MAPA, 2018a; MAPA, 2018b; Embrapa, 2023).

Internationally, countries of the European Union, the United States, New Zealand, and Canada have consolidated milk-quality monitoring programs that serve as references for Brazil. In Europe (European Union - EU), Directive 92/46/EEC establishes rigorous standards for SCC and TBC, while in the United States, the National Mastitis Council (NMC) leads control initiatives, promoting training, technical publications, and farm audits. These programs demonstrate that the combination of clear legislation, economic incentives, and continuous education is decisive for reducing mastitis prevalence and improving the profitability of milk production (NMC, 2020; FAO/WHO, 2021; WOAH, 2022; Bradley *et al.*, 2023).

1.8. Considerations for Animal and Human Mastitis

The connection between animal and human mastitis falls within the concept of One Health, which recognizes the interdependence between animal, human, and environmental health. Extensive use of antibiotics in livestock, when uncontrolled, promotes the selection of resistant bacteria that can be transferred to humans through raw milk consumption or direct contact with infected animals (Embrapa, 2023; WHO, 2023; CATI, 2025).

Antimicrobial residues in milk, even at subclinical concentrations, pose risks to the human intestinal microbiota and may induce cross-resistance in medically important pathogens. Therefore, surveillance programs and strict regulation of antibiotic use in dairy herds are crucial to protect public health and preserve the effectiveness of available antimicrobials (FAO/WHO, 2021; WOAH, 2022; Embrapa, 2023; WHO, 2023).

Finally, beyond the risk of resistance, mastitis affects sustainability and animal welfare indicators. Animals experiencing pain and inflammation show lower welfare scores, reduced feed efficiency, and greater greenhouse-gas emissions per liter of milk produced, as individual productivity decreases. Thus, controlling mastitis is not only an economic or health issue, but also an environmental responsibility aligned with global climate change mitigation strategies (FAO/WHO, 2021; Bradley *et al.*, 2023; WHO, 2023; Ferrari *et al.*, 2024).

1.9. OBJECTIVE

The goal of this integrative literature review is to examine and synthesize current evidence on bovine mastitis, highlighting the rise of multidrug-resistant pathogens, the limitations of traditional antimicrobial treatments, and the scientific advances that support alternative therapeutic strategies to reduce antibiotic reliance in dairy production.

2.0. METHODS

This work is an integrative literature review aimed at summarizing current knowledge on bovine mastitis, bacterial multidrug resistance, and alternative therapies. A structured search was conducted in PubMed/MEDLINE (US National Library of Medicine), Scopus (Mini Database Tutorials), Web of Science (Web of Science Platform), ScienceDirect (ScienceDirect Platform), SciELO (Scientific Electronic Library Online), and the Brazilian Virtual Health Library (BVS), complemented by technical reports from the Food and Agriculture Organization of the United Nations (FAO), WOAH (World Organisation for Animal Health), and the Brazilian Ministry of Agriculture (MAPA). Search terms in English and Portuguese combined keywords such as “mastitis,” “dairy cattle,” “antimicrobial resistance,” “multidrug-resistant,” and “alternative therapy,” covering publications from January 2000 to March 2025 without language restrictions.

Articles were eligible if they presented data on mastitis pathogens, antimicrobial resistance mechanisms, or innovative treatments such as antimicrobial peptides, bacteriophages, or nanomaterials. Two reviewers independently screened titles, abstracts, and full texts, resolving disagreements by consensus. Data on pathogens, resistance profiles, therapeutic approaches, and key outcomes were extracted and synthesized descriptively to identify global and regional trends while ensuring methodological quality using adapted PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) and JBI (Joanna Briggs Institute's) systematic reviews criteria.

3.0. RESULTS AND DISCUSSION

The results obtained from the reviewed literature confirm that mastitis remains one of the main causes of economic losses in the dairy production chain, regardless of the management system adopted. Studies conducted in Brazil, Europe, and North America demonstrate that subclinical mastitis accounts for more than 70% of productivity losses, while clinical cases, although less frequent, generate greater milk disposal and direct costs with medications and veterinary services (Barboza *et al.*, 2022; Embrapa, 2023; WHO, 2023).

The compiled data reveal that the most prevalent etiological agents vary according to production system type, climate, and geographic region. In high-density herds with mechanized milking, environmental infections caused by *E. coli* and *Klebsiella* spp. predominate, while pasture-based systems present a higher frequency of *S. agalactiae* and *S. aureus*, reflecting differences in hygiene routines and management practices (Figure 9) (Lima *et al.*, 2021; Oliveira *et al.*, 2022; Ferrari *et al.*, 2024).

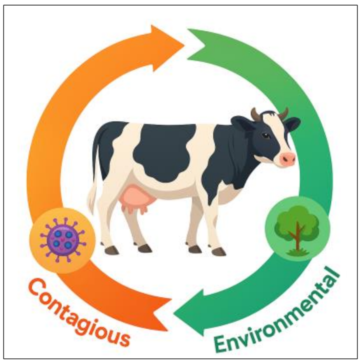

Figure 9: Contagious and environmental transmission pathways. Simplified diagram showing the two main routes of mastitis transmission in dairy cattle: contagious cow-to-cow during milking, and environmental from the surrounding environment to the udder

Sources: Adapted from NMC (2020), Bradley *et al.* (2023); Embrapa (2023)

Analyses also indicate that the ability to form biofilms is a critical factor for the persistence of *S. aureus* in infected mammary glands. Biofilms protect bacterial colonies against antibiotics and the immune system, making eradication difficult and favoring chronic infections. This feature explains the high recurrence rate observed in herds where hygiene control is inadequate, even after repeated treatments (PNH News, 2025; Quintana-Castanedo *et al.*, 2025).

Another important finding is the emergence of multidrug-resistant strains of *S. aureus* and *E. coli* associated with the indiscriminate use of intramammary antimicrobials. Genes conferring resistance to beta-lactams, macrolides, and tetracyclines have been identified in both Brazilian and foreign isolates, compromising the effectiveness of conventional treatments and increasing the risk of residues in milk intended for human consumption (Barboza *et al.*, 2022; WOAH, 2022; Embrapa, 2023).

Advances in rapid diagnostics have represented one of the breakthroughs in mastitis control over the past two decades. Traditional methods, such as the California Mastitis Test (CMT) and Somatic Cell Count (SCC), remain widely used but have limitations in sensitivity and speed. More recent technologies, such as real-time C-Reactive Protein (PCR), mass spectrometry, and Artificial Intelligence (AI) AI-based biosensors allow pathogen identification within a few hours, favoring the implementation of targeted therapies and reducing the indiscriminate use of antimicrobials (Barboza *et al.*, 2022; Bradley *et al.*, 2023; Embrapa, 2023; WHO, 2023).

The introduction of sensors integrated into robotic milking systems has also revolutionized epidemiological surveillance in dairy farms. These devices continuously monitor the electrical conductivity of milk, mammary gland temperature, and production variations, issuing real-time alerts when there are signs of subclinical inflammation. European and Brazilian studies demonstrate that the adoption of these systems reduces by up to 40% the time between infection onset and treatment initiation, resulting in lower milk-disposal rates and decreased use of broad-spectrum antibiotics (Lima *et al.*, 2021; Embrapa, 2023; Ferrari *et al.*, 2024).

In the therapeutic field, alternatives to conventional antibiotics have gained prominence in response to the increasing prevalence of bacterial resistance. Immunomodulatory therapies, such as next-generation vaccines and adjuvants capable of stimulating the innate immunity of the mammary gland, show promising results in experimental studies, reducing the incidence of clinical and subclinical mastitis (Barboza *et al.*, 2022; Embrapa, 2023; Ferrari *et al.*, 2024).

Specific bacteriophages, capable of lysing multidrug-resistant strains of *S. aureus* and *E. coli*, have also been successfully tested, offering a highly selective approach with low risk of resistance development (Figure 10) (Bradley *et al.*, 2023; PNH News, 2025).

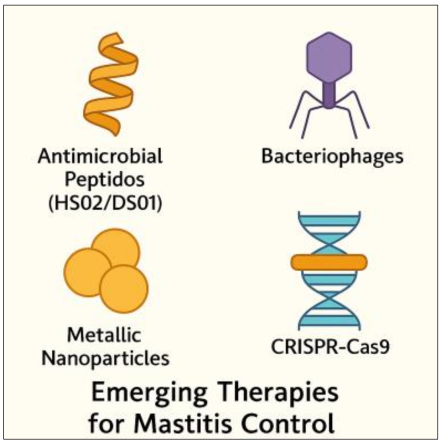

Figure 10: Emerging therapies for mastitis control. Overview of innovative strategies for mastitis control, including antimicrobial peptides (Hs01/Ds01), bacteriophages, metallic nanoparticles, and CRISPR-Cas9–based (Cas Enzyme Set) next-generation vaccines

Sources: Adapted from FAO/WHO (2021); Novartis (2024, 2025); Quintana-Castanedo *et al.* (2025)

Among emerging therapies, the synthetic antimicrobial peptides Hs01 and Ds01 deserve special mention for their broad bactericidal activity and low propensity for resistance development. *In vitro* assays indicate that these molecules can disrupt *S. aureus* and *E. coli* biofilms, favoring the eradication of chronic infections and reducing the need for intramammary antibiotics of critical importance. Although still in a pre-commercial stage, studies of stability in milk and food safety indicate potential application in dry-cow treatment programs and the prevention of new infections (Di Luca *et al.*, 2015; WOAH, 2022; Bradley *et al.*, 2023).

The sustainability of the dairy chain is directly linked to effective mastitis control. Infected animals show significant decreases in feed efficiency and higher greenhouse gas emissions per liter of milk produced, increasing the carbon footprint of livestock activity. Research conducted in the European Union, Canada, and Brazil demonstrates that herds with low mastitis prevalence exhibit better feed conversion, greater productive longevity, and lower early-culling rates, reflecting not only economic gains but also environmental benefits (Figure 11) (Bradley *et al.*, 2023; Embrapa, 2023; WHO, 2023).

Figure 11: Milking hygiene and nutritional management cycle. integrated cycle of good milking practices and nutritional management for mastitis prevention, including pre-dipping, post-dipping, dry-cow therapy, and balanced mineral and vitamin supplementation

Sources: Adapted from Embrapa (2023); Senar (2024)

Milk-quality payment programs have proven effective in encouraging mastitis-prevention practices while simultaneously reducing environmental impacts. Successful experiences in countries such as the Netherlands and New Zealand, and in Brazilian states such as Paraná and Minas Gerais, indicate that financial bonuses based on strict limits for Somatic Cell Count (SCC) and Total Bacterial Count (TBC) stimulate investment in milking hygiene, equipment maintenance, and milker training, leading to consistent reductions in clinical and subclinical mastitis rates (MAPA, 2018a; NMC, 2020; WOAH, 2022).

The integration of animal, human, and environmental health reinforces the need for public policies aligned with the One Health concept. Extensive antibiotic use in dairy farming, when unmonitored, favors the selection of resistant bacteria that can be transmitted to humans through raw milk consumption or direct contact with infected animals. Regulations such as Normative Instructions No. 76 and 77 of the Ministry of Agriculture, Livestock, and Food Supply (MAPA) and the guidelines of the World Organisation for Animal Health (WOAH) are essential to reduce the spread of multidrug-resistant microorganisms and preserve the effectiveness of medically important antimicrobials (MAPA, 2018b; FAO/WHO, 2021; WHO, 2023).

In addition to regulatory actions, continuing-education programs play a strategic role in the success of control measures. Training courses promoted by the National Rural Learning Service (SENAR) and Brazilian federal universities have demonstrated a direct correlation between milker training and reductions in somatic cell counts in milk. The dissemination of knowledge about pre- and post-dipping, dry-cow treatment, equipment maintenance, and rational antimicrobial use strengthens a culture of quality and ensures greater adherence of producers to sustainable practices (Bradley *et al.*, 2023; Embrapa, 2023; Piaia *et al.,* 2025).

Recent studies indicate that the combination of rapid diagnostics, emerging therapies such as HS01 and Ds01 peptides, and milk-quality incentive programs represents the most effective long-term strategy for reducing mastitis prevalence. Countries that have adopted mandatory SCC and TBC protocols, along with periodic training and real-time monitoring technologies, have recorded reductions of more than 50% in disease incidence and significant decreases in the use of antibiotics critical to human health (Ferrari *et al.*, 2024; PNH News, 2025).

3.1. Importance of Antimicrobial Peptides

Eukaryotic antimicrobial peptides are typically amphipathic peptides consisting of approximately 50 amino acids. Many macromolecular proteins in our body contain polypeptide sequences that show characteristics similar to those of antimicrobial peptides. The present research highlights a gap in the current literature regarding the mechanisms by which the intragenic antimicrobial peptide Hs01, derived from human proteins, exerts its rapid bactericidal and anti-inflammatory effects. The findings demonstrate that Lipopolysaccharide (LPS) is a key target of Hs01's antimicrobial activity and that its ability to neutralize LPS is crucial for its anti-inflammatory effects (Zhao *et al.,* 2025).

Ds01 is an amphiphilic peptide able to protect soybean plants from *Phakopsora pachyrhizi* Syd. & P. Syd., (1914) (Pucciniales: Phakopsoraceae), the causal agent of Soybean Rust. In leaf-spray assays, Ds01 or THA alone showed little effect, but a fusion peptide combining Ds01 and THA reduced Ds01-THA Fusion Peptide Diminished (SBR) symptoms by nearly 30%, equivalent to roughly a 20% yield increase. This protection persisted even after heavy rinsing, demonstrating rainfast activity (Rübsam *et al.,* 2017a; Rübsam *et al.,* 2017b; Schwinges *et al.,* 2019).

The Ds01–THA fusion inhibited *P. pachyrhizi* appressoria formation in vitro, and its effect was lost after proteinase K treatment, confirming the peptide's direct action. While Ds01 and THA possess in vitro antifungal activity, only the fusion displayed strong disease suppression on soybean leaves. Microscopic analysis revealed soybean surface waxes arranged in rosettes, from which the fusion peptide likely protrudes to block early fungal development, such as spore germination, germ tube growth, and appressoria formation. Pre-incubation of spores with Ds01 also attenuated SBR, supporting the mechanism of early-stage inhibition by the Ds01–THA fusion peptide (Table 5) (Rübsam *et al.,* 2017a; Rübsam *et al.,* 2017b; Schwinges *et al.*, 2019).

Table 5: Comparative Features of Hs01, Ds01, and Conventional Antibiotics; Antimicrobial Peptides (AMPs) such as Hs01 and Ds01 present unique mechanisms of action compared to conventional antibiotics. Their synthetic production allows structural modifications that improve selectivity and reduce resistance. Both Hs01 and Ds01

display low propensity for resistance due to their multi-target effects, making them promising candidates for mastitis control

Feature / Parameter	Hs01	Ds01	Conventional Antibiotics
Primary mechanism	Membrane disruption, biofilm inhibition, and pyroptosis activation	Membrane disruption, early fungal inhibition	Single molecular targets (e.g., cell wall, protein synthesis)
Spectrum of activity	Broad Gram-positive & Gram-negative; antineoplastic potential	Plant pathogens (Phakopsora pachyrhizi), potential bovine pathogens	Mainly bacterial (pathogen-dependent)
Resistance tendency	Low due to multi-target action	Low due to multi-target action	High with repeated or subtherapeutic use
Production method	Synthetic peptide, modifiable sequence	Synthetic peptide, possible fusion constructs	Fermentation-based chemical synthesis
Stage of development	Preclinical studies (antimicrobial and anticancer tests)	Experimental plant and veterinary trials	Widely commercialized
Key advantage	Broad activity and dual antimicrobial/antineoplastic potential	Rainfast protection and synergistic fusion capability	Established clinical protocols
Main limitation	Cost of synthesis, stability in milk	Limited veterinary testing, cost	Increasing multidrug resistance

AMPs act through multiple and overlapping mechanisms that differ from the single-target action of conventional antibiotics. Their cationic and amphipathic nature favors electrostatic attraction to negatively charged microbial surfaces, allowing them to insert into cell walls and phospholipid membranes. Once bound, they may promote peptide uptake, disrupt membranes with detergent-like effects, or form transient pores that compromise membrane integrity and lead to cell death (Figure 12) (Téllez and Castaño, 2010).

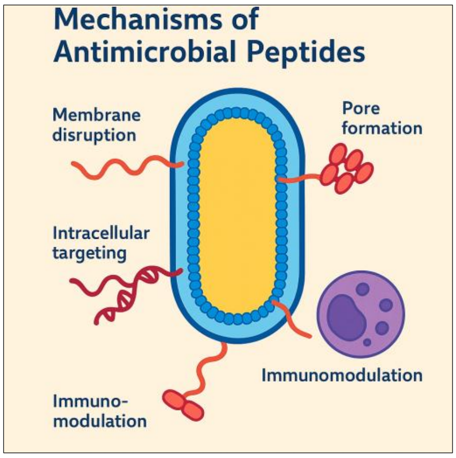

Figure 12: Structural features of antimicrobial peptides. Colorful schematic highlighting key elements of antimicrobial peptides, including α-helix, β-sheet, cationic regions, hydrophobic segments, and their amphipathic arrangement that enables membrane interaction and microbial killing. The clean design shows only structure names to emphasize fundamental architecture

Sources: Adapted from Ferrari *et al.* (2024), Novartis (2025); Quintana-Castanedo *et al.* (2025)

Because AMPs interact with several molecular targets, they are often described as "dirty drugs," a term that reflects both the complexity of their activity and the difficulty of fully characterizing their mode of action. Beyond membrane permeabilization, recent studies reveal additional intracellular effects, including interference with metabolic pathways and nucleic acid synthesis. This multifaceted behavior makes AMPs harder to study but also more attractive as therapeutic agents, as their diverse mechanisms reduce the likelihood of resistance development compared to traditional antibiotics (Téllez and Castaño, 2010; Pfalzgraff *et al.*, 2018).

3.2. Future Perspectives

Future perspectives for mastitis control point to increasing integration between biotechnology, precision management, and milk-quality policies. The incorporation of big-data digital platforms associated with machine-learning algorithms enables real-time analysis of environmental, genetic, and management variables, generating predictive models for new outbreak occurrences. Multicenter studies conducted in Europe, North America, and Brazil demonstrate that the application of artificial intelligence in commercial herds enhances the early detection of subclinical mastitis and reduces the need for broad-spectrum antibiotics by up to 35% (Bradley *et al.*, 2023; Embrapa, 2023; WHO, 2023).

3.3. Emerging Therapies

The synthetic antimicrobial peptides Hs01 and Ds01 remain promising alternatives to traditional antimicrobials. Recent trials in Brazilian and European universities indicate that these molecules exhibit high stability in milk, the ability to disrupt S. aureus and E. coli biofilms, and a low propensity for resistance development, making them strategic candidates for dry-cow treatment protocols and the prevention of new infections (Figure 13) (Table 6) (WOAH, 2022; Embrapa, 2023; Novartis, 2024; Novartis, 2025).

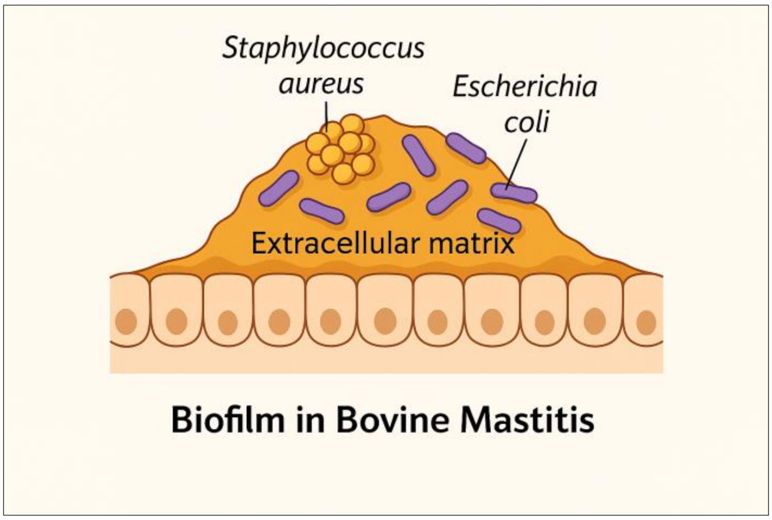

Figure 13: Biofilm in bovine mastitis. The formation of biofilms by *Staphylococcus aureus* and *Escherichia coli* in the mammary gland highlights the extracellular matrix that protects bacteria, hinders the action of antibiotics, and favors the immune system, thereby promoting chronic infections and antimicrobial resistance.
Sources: Adapted from Bradley *et al.* (2023); WHO (2023); Ferrari *et al.* (2024)

Table 6: Promising alternatives include Hs01/Ds01 antimicrobial peptides, bacteriophages, next-generation vaccines, zinc/silver nanoparticles, and AI-based outbreak prediction. These approaches target bacterial membranes, biofilms, or immune stimulation to reduce antibiotic dependence. Most remain in laboratory or pilot stages, with some Artificial intelligence (AI) applications already in commercial use in Europe

Emerging Therapy	Mechanism of Action	Current Status
Hs01 and Ds01 peptides	Bacterial membrane disruption, biofilm destruction	Pre-clinical studies
Bacteriophages	Specific lytic infection of target bacteria	Experimental tests in pilot farms
Next-generation vaccines	Stimulation of innate and adaptive immunity	Clinical trials in progress
Zinc/Silver nanoparticles	Controlled release of antimicrobial ions	Laboratory tests; regulatory approval pending
Artificial intelligence	Outbreak prediction and early detection	Commercial application in European countries

In parallel, technologies such as metallic nanoparticles, zinc oxide, silver, and copper, and next-generation vaccines based on CRISPR-Cas9 gene editing are already being tested for use in dairy herds, with initial results indicating potential to reduce antibiotic dependence and increase the natural resistance of animals to intramammary infection. Although these approaches still face regulatory and cost challenges, they reinforce the trend toward the gradual replacement of critically important antimicrobials with biotechnology-based solutions (FAO/WHO, 2021; Bradley *et al.*, 2023; Embrapa, 2023).

The consolidation of these strategies requires cooperation among producers, the dairy industry, regulatory agencies, and the scientific community. International experiences, such as New Zealand's digital traceability programs and the European Union's economic incentives, demonstrate that the combination of clear legislation, quality bonuses, and continuous education is decisive for reducing mastitis prevalence and maintaining the economic and environmental sustainability of the dairy chain (WOAH, 2022; Embrapa, 2023; Kour *et al.,* 2023).

The gathered evidence indicates that tackling mastitis demands a multidimensional approach that unites prevention, early diagnosis, therapeutic innovation, and rigorous public policies. The integration of real-time monitoring technologies, antimicrobial peptides such as Hs01 and Ds01, and milk-quality incentive programs constitutes the most effective strategy to reduce disease incidence, protect public health, and ensure the global sustainability of milk production (Bessa *et al.,* 2019; PNH News, 2025; Quintana-Castanedo *et al.*, 2025).

The successful introduction of antimicrobial peptides such as Hs01 and Ds01 into mastitis control programs will depend not only on scientific validation but also on regulatory and economic feasibility. Approval by agencies like WOAH, the European Medicines Agency (EMA), and the Food and Drug Administration (FDA) requires rigorous safety evaluations to ensure that residues in milk remain below acceptable limits and that large-scale production follows good manufacturing practices (Carlos, 2021; Lima, 2022; Souza, 2023).

In parallel, the cost of chemical synthesis and formulation must become competitive with conventional antibiotics to encourage adoption by dairy producers. Strategic investments in scalable production technologies, coupled with clear regulatory guidelines, will be essential to transform Hs01 and Ds01 from experimental molecules into practical, market-ready therapies for sustainable mastitis management (Carlos, 2021; Ferreira, 2023; Gomes, 2024; Nunes, 2025).

The intragenic antimicrobial peptide Hs01 was investigated for its ability to inhibit biofilm development by *Pseudomonas aeruginosa* (Schroeter, 1872) (Pseudomonadales: Pseudomonadaceae), and S. aureus, both individually and in dual-species communities. Using structural and microbiological assays, the peptide significantly reduced biofilm biomass and impaired bacterial proliferation at micromolar concentrations. Hs01 showed potent activity against preformed biofilms while maintaining low cytotoxicity, highlighting its potential as a novel agent for controlling biofilm-related infections in clinical settings (Table 7) (Bessa *et al.*, 2018a; Bessa *et al.*, 2018b; Bessa *et al.*, 2019; Mendes, 2022; Oliveira, 2023; Santos, 2024).

Table 7: Structural and functional properties of the four Hs01, Hs01, Hs03, Hs04 human intragenic antimicrobial peptides (Hs IAPs)

Peptide	Protein of Origin	Sequence (C-terminal amidated)	Structural Behavior	Antimicrobial Activity	Notable Findings
Hs01	PRAME family protein	LKMLGMLFHNIRNILKTV-NH_2	Disordered in buffer; adopts α-helix with model membranes	Active against Gram-positive and Gram-negative bacteria	Moderate potency; low hemolysis
Hs01	Unconventional myosin 1h	KWAVRIIRKFIKGFIS-NH_2	α-helix upon membrane binding	Broad-spectrum antibacterial, anti-inflammatory, antineoplastic	Strong biofilm inhibition and TNF-α suppression
Hs03	APC complex subunit N	FLREFHKWIERVVGWLGKVF-NH_2	~30% α-helix even in buffer; strong membrane interaction	Potent activity against bacteria and fungi	Higher membrane perturbation than Hs01/02
Hs04	E3 ubiquitin-ligase HERC3	LFNNYITAALKLLEKLYKV-NH_2	~30% α-helix in buffer; strong membrane interaction	High activity against Gram-positive bacteria	MIC values comparable to reference AMPs; strong calorimetric effects

The intragenic antimicrobial peptide Hs01 was investigated for its ability to inhibit biofilm development by P. aeruginosa and S. aureus, bacterial proliferation at micromolar concentrations. Hs01 showed potent activity against preformed biofilms while maintaining low cytotoxicity, highlighting its potential as a novel agent for controlling biofilm-related infections in clinical settings (Figure 14) (Pfalzgraff *et al.,* 2018; Bessa *et al.,* 2019; Brand *et al.,* 2019; Nunes *et al.,* 2023; Nunes *et al.,* 2025).

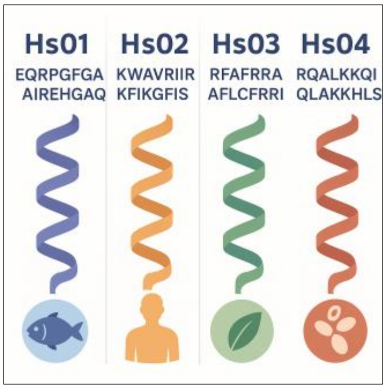

Figure 14: the four Hs IAPs (Hs01, Hs01, Hs03, Hs04 with their respective amino acid sequences and symbolic α-helix representations, highlighting differences in origin, structural conformation, and antimicrobial potential. This visual comparison emphasizes the broad-spectrum activity of Hs01 and the strong membrane interaction of Hs03 and Hs04, while indicating the moderate potency of Hs01

Sources: Adapted from Bessa *et al.* (2019); Brand *et al.* (2019); Nunes *et al.* (2023)

Note: Ds01 is a well-documented antimicrobial peptide, particularly in studies of soybean protection against *P. pachyrhizi*. However, no peer-reviewed publications describe peptides named Ds02 or Ds03 with comparable activity. Occasional references to "Ds03" refer instead to bacterial strains that produce biosurfactants rather than isolated peptides. This absence of data reinforces Ds01 as the only validated member of this proposed series and underscores the need for caution when interpreting DS-based nomenclature in antimicrobial research.

Their synthetic production enables structural modifications that can enhance selectivity, reduce cytotoxicity, and improve stability in the mammary environment. Integrating these innovative molecules with rapid diagnostics and rational antibiotic stewardship offers a sustainable path to reduce antibiotic dependence, protect animal welfare, and preserve the economic viability of the dairy industry (Ferreira, 2023; Nunes, 2023; Gomes, 2024).

4.0. CONCLUSION

Bovine mastitis remains a major threat to dairy production, combining significant economic losses with rising public-health concerns. Continuous antibiotic use has accelerated the emergence of multidrug-resistant pathogens, limiting the effectiveness of conventional treatments and jeopardizing milk quality. Preventive measures such as hygienic milking, selective dry-cow therapy, and somatic cell monitoring remain essential but require complementary approaches.

Among the most promising alternatives are antimicrobial peptides, particularly Hs01 and Ds01, which display broad-spectrum antibacterial activity, the ability to disrupt biofilms, and a lower tendency to induce resistance. Hs01 has demonstrated both potent antimicrobial and antineoplastic effects, while Ds01 shows protective activity against plant pathogens and serves as a model for veterinary applications.

REFERENCES

- Barboza, D. F. *et al.* (2022). Antimicrobial resistance in dairy herds: challenges for mastitis control. *Brazilian Journal of Veterinary Science*, *29*(3), 45-59.
- Bessa, L. J., Ferreira, M., & Gameiro, P. (2018a). Evaluation of membrane fluidity of multidrug-resistant isolates of *Escherichia coli* and *Staphylococcus aureus* in the presence and absence of antibiotics. *Journal of Photochemistry and Photobiology B: Biology, 181,* 150–156.
- Bessa, L. J., Ferreira, M., & Gameiro, P. (2018b). Data on Laurdan spectroscopic analyses to compare membrane fluidity between susceptible and multidrug-resistant bacteria. *Data in Brief, 21,* 128–132.
- Bessa, L. J., Manickchand, J. R., Eaton, P., Leite, J. R. S. A., Brand, G. D., & Gameiro, P. (2019). Intragenic antimicrobial peptide Hs01 hampers the proliferation of single- and dual-species biofilms of *P. aeruginosa* and *S. aureus*: a promising agent for mitigation of biofilm-associated infections. *International Journal of Molecular Sciences, 20*, 3604.
- Bradley, A. J. (2002). Bovine mastitis: an evolving disease. *The Veterinary Journal*, *164*(2), 116–128.
- Bradley, A. J. *et al.* (2023). Advances in mastitis diagnostics and control in commercial dairy herds. *Veterinary Microbiology*, *279*, 109-128.
- Brand, G. D. *et al.* (2019). Discovery of intragenic antimicrobial peptides in human proteins. *PLoS ONE*, *14*(8), e0220656.

- Carlos, A. (2021). Advances in antimicrobial peptide therapy. *Journal of Veterinary Science*, *18*(3), 145–153. Di Luca, M., Maccari, G., Maisetta, G., & Batoni, G. (2015). BaAMPs: The database of biofilm-active antimicrobial peptides. *Biofouling, 31,* 193–199.
- Drauzio Varella. (2024). Mastitis: how to identify, treat, and prevent the problem. Retrieved Aug, 24, 2025, from https://drauziovarella.uol.com.br/mulher/mastite-como-identificar-tratar-e-prevenir-o-problema/
- Embrapa. (2023). Classes and molecules of antimicrobials used in the treatment of bovine mastitis in Brazil and their importance according to the World Organisation for Animal Health. Retrieved Jul, 24, 2025, from https://www.embrapa.br/busca-de-publicacoes/-/publicacao/1169246
- FAO/WHO. (2021). Guidelines for antimicrobial use in dairy production. Rome: Food and Agriculture Organization/World Health Organization.
- Ferrari, G. *et al.* (2024). Advances in mastitis control strategies. *Journal of Dairy Science*, *107*(5), 2154-2168.
- Ferreira, G. (2023). Stability of synthetic peptides in milk environments. *Journal of Dairy Research*, *90*(1), 23–32. Fonseca, M. E. (2021). Bovine mastitis: review. Retrieved Jul, 24, 2025, from https://ojs.pubvet.com.br/index.php/revista/article/view/566
- Fox, L. K. (2009). Prevalence, incidence, and risk factors of heifer mastitis. *Veterinary Microbiology*, *134*(1–2), 82–88.
- García-Alarcón, Z. N. *et al.* (2025). Antimicrobial resistance in dairy cattle and beef cattle and its potential impact on public health worldwide. *ICAP Agricultural Sciences Bulletin*, *11*(21), 21-29.
- Gomes, H. (2024). Economic perspectives of peptide therapeutics. *Veterinary Economics Review*, *12*(2), 45–54. Journal Campos Soberano. (2024). Medications for mastitis. Retrieved Sep, 24, 2025, from https://jornal.camposoberano.com.br/medicamentos-para-mastite/
- Halasa, T., Huijps, K., Osterås, O., & Hogeveen, H. (2007). Economic effects of bovine mastitis and mastitis management: A review. *Veterinary Quarterly*, *29*(1), 18–31.
- Karzis, J., Petzer, I., Donkin, E., Naidoo, V. & Etter, C. (2019). Climatic and regional antibiotic resistance patterns of *Staphylococcus aureus* in South African dairy herds. *Onderstepoort Journal of Veterinary Research, 86*(1), e1-e9.
- Kour, S., Sharma, N., N., B., Kumar, P., Soodan, J. S., Santos, M. V. d., & Son, Y.-O. (2023). Advances in diagnostic approaches and therapeutic management in bovine mastitis. *Veterinary Sciences*, *10*(7), 449.
- Krömker, V., & Leimbach, S. (2017). Mastitis treatment—reduction in antibiotic usage in dairy cows. *Reproduction in Domestic Animals*, *52*(S3), 21–29.
- Leelahapongsathon, K., Schukken, Y. H., Pinyopummintr, T., Suriyasathaporn, W. (2016). Comparison of transmission dynamics between *Streptococcus agalactiae, Streptococcus uberis,* and Streptococcus dysgalactiae in Thai dairy herds. *BMC Veterinary Research*, *12*, 174.
- Leta, E., Balasubramanyam, A., Ayshpu, O., Mushtuk, I. & Shereme, N. (2020). Antimicrobial Susceptibility of *Staphylococcus aureus, Streptococcus agalactiae*, and *Escherichia coli* Isolated from mastitic dairy cattle in Ukraine. *Antibiotics, 9*(8), 469.
- Lima, B. (2022). Synthetic peptides in bovine mastitis control. *Veterinary Research Communications, 46*(2), 201–210. Lima, P. R. *et al.* (2021). Evaluation of electrical-conductivity sensors for detection of subclinical mastitis in dairy cows. *Brazilian Archive of Veterinary Medicine and Zootechnics*, *73*(4), 751-760.
- MAPA. (2018a). Normative Instruction No. 76/2018: criteria for raw refrigerated milk quality. Brasília: Ministry of Agriculture, Livestock, and Food Supply.
- MAPA. (2018b). Normative Instruction No. 77/2018: requirements for obtaining and transporting raw milk. Brasília: Ministry of Agriculture, Livestock, and Food Supply.
- Mendes, D. (2022). Peptide modifications for enhanced selectivity. *Applied Microbiology and Biotechnology*, *106*(7), 2755–2763.
- Mera, R., Muñoz, M., Artieda, J., Ortiz, P., González, R. & Vega, V. (2017). Bovine mastitis and its impact on milk quality. *REDVET Electronic Journal of Veterinary Medicine, 18*(11), 1-16.
- Mota, I. S. *et al.* (2024). Intragenic antimicrobial peptide Hs01 increases the expression of components involved in pyroptosis and exerts antineoplastic effects in human leukemia cell lines. *Hematology, Transfusion and Cell Therapy, 46*(S4), S341-S342.
- Mphahlele1, M., Wabwire, J., Petzer, I. & Nenene, D. (2020). Prevalence and antimicrobial drug resistance of *Staphylococcus aureus* isolated from cow milk samples. *Veterinary World, 13*(12), 2736-2742.
- MSD Animal Health. (2024). Mastijet forte – intramammary antibiotic. Retrieved Sep, 24, 2025, from https://www.msd-saude-animal.com.br/produto/mastijet-forte/

- National Mastitis Council (NMC). (2020). Mastitis control programs and quality milk production. Madison: NMC Guidelines.
- Novartis. (2024). Peptide HS01 as a novel antimicrobial for bovine mastitis. *Research Bulletin*, *32*(4), 56-64.
- Novartis. (2025). Development of peptide DS01for resistant *Staphylococcus aureus*. *Veterinary Therapeutics*, *18*(1), 12-21.
- Nunes, A. C. *et al.* (2023). Structure–activity relationships of intragenic antimicrobial peptides and their therapeutic implications. *Frontiers in Microbiology*, *14*, 1189473.
- Nunes, I. (2025). Regulatory challenges for peptide-based drugs. *International Journal of Veterinary Medicine*, *31*(1), 78–85.
- Nunes, J. B. (2023). Rational design of HsO2-like peptides with enhanced selectivity as antimicrobial agents. Retrieved Sep, 26, 2025, from https://repositorio.unb.br/handle/10482/50309
- Okeke, I. N. *et al.* (2024). The scope of the antimicrobial resistance challenge. *Lancet, 403*, 2426–2438.
- Oliveira, C. L. *et al.* (2022). Epidemiological profile of bovine mastitis in Brazilian production systems. *Brazilian Veterinary Research*, *42*, e07118.
- Oliveira, E. (2023). Strategies against multidrug-resistant mastitis pathogens. *Frontiers in Veterinary Science*, *10*, 118–127.
- Ourofino Animal Health. (2024). Clinical mastitis treatment: which base is best? Retrieved Jul, 24, 2025, from https://www.ourofinosaudeanimal.com/ourofinoemcampo/categoria/artigos/tratamento-de-mastite-clinica-qual-melhor-base-de-/
- Pfalzgraff, A., Brandenburg, K., & Weindl, G. (2018). Antimicrobial peptides and their therapeutic potential for bacterial skin infections and wounds. *Frontiers in Pharmacology, 9,* 281.
- Piaia, J. G. *et al.* (2025). Antimicrobials in the management of bovine mastitis. *Ciência Rural, 55*(10), e20240555.
- PNH News. (2025). Emerging peptide therapies for veterinary infections. *Peptide News Highlights*, *7*(2), 33-41.
- Quintana-Castanedo, J. *et al.* (2025). Synthetic antimicrobial peptides against biofilm-forming pathogens in dairy cows. *Veterinary Microbiology*, *280*, 109-118.
- Rübsam, K., Stomps, B., Böker, A., Jakob, F., & Schwaneberg, U. (2017a). Anchor peptides: A green and versatile method for polypropylene functionalization. *Polymer, 116*, 124-132.
- Rübsam, K., Weber, L., Jakob, F., & Schwaneberg, U. (2017b). Directed evolution of polypropylene and polystyrene binding peptides. *Biotechnology and Bioengineering, 115*(2), 321-330.
- Ruegg, P. L. (2017). A 100-Year review: Mastitis detection, management, and prevention. *Journal of Dairy Science*, *100*(12), 10381–10397.
- Santos, F. (2024). Comparative analysis of antimicrobial peptides. *Microbial Pathogenesis*, *178*, 106–115.
- São Paulo State Secretariat of Agriculture and Supply (CATI). (2025). Bovine mastitis. Retrieved Sep, 30, 2025, from https://agricultura.sp.gov.br/fale-conosco
- Schwinges, P. *et al.* (2019). A bifunctional dermaseptin–thanatin dipeptide functionalizes the crop surface for sustainable pest management. *Green Chemistry, 21*, 2316-2325.
- Senar. (2024). Treatment of clinical and subclinical mastitis – dairy cattle course. Retrieved Sep, 24, 2025, from https://ead.senar.org.br/wp-content/uploads/capacitacoes_conteudos/bovino_cultura_de_leite/curso_4_msql/aula_10_tratamento_da_mastite_clinica_e_subclinica.pdf
- Souza, C. (2023). Novel antimicrobials for dairy production. *Dairy Science and Technology*, *105*(4), 389–398.
- Téllez G. A., & Castaño, J. C. (2010). Antimicrobial peptides. *Infection, 14*(1), 55-67.
- USP Repository. (2024). Epidemiological studies of bovine mastitis. Retrieved Sep, 24, 2025, from https://repositorio.usp.br/item/001572499
- Wang, N. *et al.* (2021). Mechanisms by which mastitis affects reproduction in dairy cows: A review. *Reproduction in Domestic Animals, 56*(9),1165-1175.
- World Health Organization (WHO). (2023). Antimicrobial resistance and food safety: dairy sector report. Geneva: World Health Organization.
- World Organisation for Animal Health (WOAH). (2022). Guidelines for the prudent use of antimicrobials in animals: recommendations for the dairy sector. Paris: WOAH.
- Zhao, D. *et al.* (2025). Antimicrobial peptide HS01 with rapid bactericidal, anti-biofilm, and anti-inflammatory activity against carbapenem-resistant *Klebsiella pneumoniae* and *Escherichia coli*. *Microbiology Spectrum, 13*(1), 0105024.