ANA JÚLIA SILVA MOREIRA

EXPLORING LYNRONNE-1 AND COMBINATIONAL THERAPY AS ALTERNATIVES TO ANTIBIOTICS FOR CONTROLLING BOVINE MASTITIS

Dissertation submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Hilário Cuquetto Mantovani

Co-adviser: Maria Aparecida Scatamburlo Moreira

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To God.

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"E lá vou eu, nas minhas tentativas, às vezes meio cegas, às vezes meio burras, tentar acertar os passos.."
(Caio Fernando Abreu)

ABSTRACT

MOREIRA, Ana Júlia Silva, M.Sc., Universidade Federal de Viçosa, May, 2022. Exploring lynronne-1 and combinational therapy as alternatives to antibiotics for controlling bovine mastitis. Adviser: Hilário Cuquetto Mantovani. Co-adviser: Maria Aparecida Scatamburlo Moreira.

Bovine mastitis stands as a significant challenge in the dairy sector, driving high economic costs and extensive antibiotic use, which fuels antimicrobial resistance concerns. In response, this study investigates the efficacy of antimicrobial peptides (AMPs) and combinational treatments as potential alternatives to antibiotics for controlling mastitis pathogens. Five AMPs: lynronne-1 (lyn-1), lynronne-2 (lyn-2), Bovicin HC5 AMP 660, and AMP 1043; alongside bioactive compounds disodium ethylenediaminetetraacetic acid (EDTA) and glycerol monolaurate (GML), were assessed against 35 mastitis-causing pathogens, including prominent strains such as *Staphylococcus aureus*, *Staphylococcus chromogenes, Streptococcus agalactiae, Streptococcus dysgalactiae, Klebsiella pneumoniae* and *Escherichia coli*. Lyn-1 and EDTA emerged as the most effective treatments, exhibiting broad-spectrum antimicrobial activity with substantial reductions in bacterial optical density. Combinatorial assays revealed synergistic interactions, as lyn-1-EDTA combination demonstrated superior antimicrobial activity at lower concentrations than individual compounds. This combination proved bactericidal, significantly reducing bacterial populations within a short timeframe. The combination exhibited non-hemolytic behavior, although cytotoxic effects were observed at concentrations above the minimal inhibitory concentration (MIC) in epithelial bovine mammary alveolar (MAC-T) cells. In parallel, the capacity of lyn-1 to reduce bacterial biofilm formation and disrupt established biofilms was assessed, alongside its capacity to reduce adhesion and invasion of mastitis pathogens to MAC-T cells. Moreover, a clinical trial evaluated the intramammary treatment with Lyn-1, embedded in chitosan hydrogel, in Holstein cows with high somatic cell counts (SCC). While inducing a transient increase in SCC, the treatment showed comparable efficacy to the antibiotic control group in terms of SCC, total bacterial count, and milk composition, with no detectable antimicrobial residues. These findings underscore the therapeutic potential of lyn-1 and its combinational treatments in controlling mastitis pathogens, offering insights into the development of antibiotic-free formulations for udder infection management and addressing concerns regarding antibiotic resistance in the dairy industry.

Key words: bovine mastitis, antimicrobial peptides, alternative therapy.

RESUMO

MOREIRA, Ana Júlia Silva, M.Sc., Universidade Federal de Viçosa, maio, 2022. Explorando o lynronne-1 e a terapia combinatória como alternativas aos antibióticos para o controle da mastite bovina. Orientador: Hilário Cuquetto Mantovani. Co-orientador: Maria Aparecida Scatamburlo Moreira.

A mastite bovina representa um desafio significativo no setor leiteiro, gerando altos custos econômicos e amplo uso de antibióticos, o que gera sérias preocupações com a resistência antimicrobiana. Em resposta, este estudo investiga a eficácia de peptídeos antimicrobianos (AMPs) e tratamentos combinados como alternativas potenciais aos antibióticos para controlar patógenos da mastite bovina. Cinco AMPs: lynronne-1 (lyn-1), lynronne-2 (lyn-2), Bovicin HC5, AMP 660 e AMP 1043; juntamente com os compostos bioativos (etilenodiaminotetraacetato dissódico (EDTA) e monolaurato de glicerol (GML; foram avaliados contra 35 patógenos causadores de mastite, incluindo cepas proeminentes como *Staphylococcus aureus, Staphylococcus chromogenes, Streptococcus agalactiae, Streptococcus dysgalactiae, Klebsiella pneumoniae* e *Escherichia coli*. Lyn-1 e EDTA surgiram como os tratamentos mais eficazes, exibindo atividade antimicrobiana de amplo espectro com reduções substanciais na densidade óptica bacteriana. Ensaios combinatórios revelaram interações sinérgicas, visto que a combinação Lyn-1-EDTA demonstrou atividade antimicrobiana superior em concentrações mais baixas do que compostos individuais. Esta combinação mostrou-se bactericida, reduzindo significativamente as populações bacterianas em curto período de tempo. Importante destacar que a combinação exibiu comportamento não hemolítico, embora efeitos citotóxicos tenham sido observados em concentrações acima da concentração inibitória mínima (CIM) em células alveolares mamárias bovinas (MAC-T). Em paralelo, foi avaliada a capacidade de lyn-1 em reduzir a formação de biofilme bacteriano e desestabilizar biofilmes estabelecidos, além de sua capacidade em reduzir a adesão e invasão de patógenos da mastite à linhagem MAC-T. Além disso, um ensaio clínico avaliou o tratamento intramamário com Lyn-1, incorporado em hidrogel de quitosana, em vacas holandesas com contagens de células somáticas (CCS) elevadas. Embora tenha induzido um aumento transitório na CCS, o tratamento mostrou eficácia comparável ao grupo controle de antibióticos em termos de CCS, contagem total de bactérias e composição do leite, sem resíduos antimicrobianos detectáveis. Esses achados destacam o potencial terapêutico de Lyn-1 e seus tratamentos combinados no controle de patógenos da mastite, oferecendo insights para o desenvolvimento de formulações livres de antibióticos para o manejo de infecções da glândula

mamária e abordando preocupações relacionadas à resistência aos antibióticos na indústria leiteira.

Palavras-chave: mastite bovina, peptídeos antimicrobianos, terapia alternativa.

SUMMARY

GENERAL INTRODUCTION

Bovine mastitis, an inflammatory condition triggered by microbial infections, poses a significant challenge to dairy farmers globally. Its prevalence, coupled with diagnostic and treatment complexities, makes it a persistent issue. Considered the costliest ailment disease affecting dairy cattle worldwide, mastitis accounts for over 70% of milk production reduction, 9% of discarded milk post-treatment, 7% of veterinary service expenses, and 14% of premature culling (Ashraf *et al.*, 2018). In the United States alone, mastitis leads to an annual industry loss of $2 billion, with an average cost of $192.36 ± 8.90 per clinical case, not covering expenses such as diagnostics, veterinary services, decreased milk yield, and premature culling (Oliveira, Hulland and Ruegg, 2013; de Campos *et al.*, 2023). When analyzing these indirect losses, this value can reach $444 per case (Rollin, Dhuyvetter and Overton, 2015).

Intramammary infections can lead to two main forms of mastitis: clinical mastitis, characterized by visible abnormalities in milk and udder, and subclinical mastitis, which lacks detectable changes in mammary secretions (Ruegg, 2024). While the majority (>85%) of clinical mastitis (CM) cases are non-severe, antibiotics are commonly used for treatment, often exceeding recommended durations (Ruegg, 2018, 2020). In the United States, seven approved intramammary (IMM) antibiotics target primarily contagious Gram-positive pathogens responsible for severe CM cases (Ruegg, 2020, 2021). However, treatment outcomes are significantly influenced by factors such as the underlying cause, lactation stage, pathology severity, and host immune response. Current therapies are ineffective against opportunistic environmental pathogens like *Escherichia coli*, environmental streptococci, and *Klebsiella* species (Oliveira and Ruegg, 2014; Ruegg, 2018; Fuenzalida and Ruegg, 2019; Cheng and Han, 2020).

Moreover, the use of antibiotics throughout the milk production chain can pose significant risks to human health by fostering the selection of bacteria carrying resistance genes, which can then be transferred to other strains or species (Rubiola *et al.*, 2020). The World Health Organization (2017) has identified antimicrobial resistance as a major global threat to public health. In animal husbandry, a great amount of antibiotics is often administered to healthy animals for disease prevention, creating potential reservoirs of resistant bacteria capable of disseminating resistance genes. Therefore, there is an urgent need for novel and effective

therapeutic strategies to control microbial infections in livestock that are safe and with little impact on the selection of resistant organisms (Cheng and Han, 2020).

In recent years, there has been a growing scientific interest in utilizing antimicrobial peptides (AMPs) as potential chemotherapeutic agents against bacterial pathogens (Guilhelmelli *et al.*, 2013; Kang *et al.*, 2017; Chen and Lu, 2020; Luong, Thanh and Tran, 2020; Mulukutla *et al.*, 2024). These peptides, produced by bacteria themselves, often exhibit high potency, low toxicity, and good stability. Importantly, AMPs typically target different cellular components compared to conventional antibiotics, thereby reducing the likelihood of cross-resistance (Yu *et al.*, 2018). Resistance to AMPs is generally rare or occurs at a low rate. Furthermore, their gene-encoded nature renders them amenable to genetic manipulation and facilitates novel drug design. (Lai and Gallo, 2009; Kim, Rajasekaran and Shin, 2017; Pletzer, Mansour and Hancock, 2018). This study aimed to evaluate peptides with antimicrobial activity as potential alternative treatments for bovine mastitis compared to traditional antibiotics.

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LITERATURE REVIEW

TACKLING BOVINE MASTITIS: CHALLENGES AND INNOVATIONS

BOVINE MASTITIS AND ITS ECONOMIC IMPACT

Mastitis is an inflammatory disease of the mammary gland caused by harmful microorganisms. This condition can significantly reduce milk production and result in the rejection of milk in dairy production due to the presence of antibiotic residues or high somatic cell count. In addition to lowering productivity, mastitis can also decrease profitability by reducing production performance, increasing veterinary, medication, and management costs, reducing the sale value of cows, and increasing replacement costs due to early disposal. As a result, bovine mastitis is considered the costliest disease in the sector, as indicated by several studies (Ashraf *et al.*, 2018; Halasa *et al.*, 2007; Oliveira *et al.*, 2013; Rodriguez *et al.*, 2024; Rollin *et al.*, 2015).

For example, Halasa *et al.* (2007) estimated an average cost of US$393 per case after analyzing peer-reviewed studies over 17 years, and Rollin *et al.* (2015) increased this estimate to US$444 per clinical case, including both direct and indirect costs associated with the occurrence of mastitis within the first 30 days of lactation (Fig. 1). On the other hand, a case of subclinical mastitis may cause a production loss that ranges between 10% and 20% less milk over a lactation per cow per year, as they reduce milk quality and volume. The greatest economic losses are attributed to high bulk tank SCC, resulting in loss of quality premiums and milk production per cow (Ruegg, 2012). The overall production loss caused by subclinical mastitis is estimated at $110/case annually for the average US dairy farm (Ruegg, 2012). Mastitis also causes additional losses due to animal death, decreased genetic gain, and reductions in reproductive efficiency of animals (Ruegg, 2012). In the United States, mastitis is estimated to cost the dairy industry around $2 billion annually (Cobirka *et al.*, 2020).

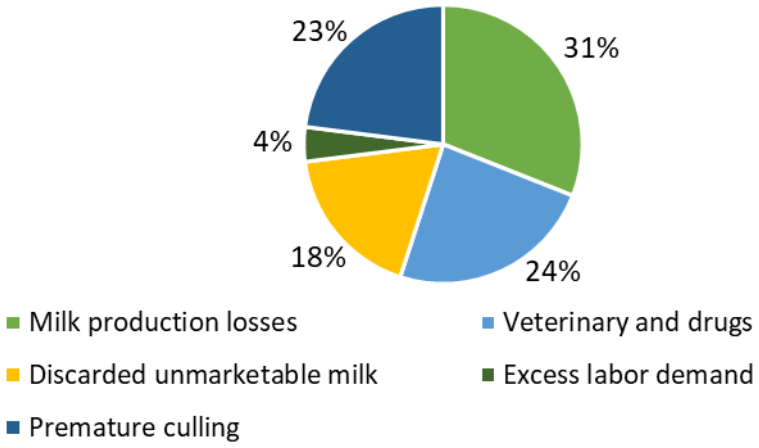

Figure 1: Breakdown of costs for clinical mastitis according to Heikkilä *et al.* (2012).

Classification and Causes of Bovine Mastitis

Bovine mastitis agents are divided into two categories: contagious and environmental (Fig. 2a). Contagious mastitis involves pathogen reservoirs in the infected cow's udder, with transmission occurring during milking and cow management. The primary causative agents are *Staphylococcus aureus* and *Streptococcus agalactiae*, resulting in chronic subclinical infections with occasional clinical outbreaks. Environmental mastitis, on the other hand, is characterized by short-term clinical infections acquired from the cow's environment, caused by strains of *Escherichia coli*, *Klebsiella* spp., *Streptococcus dysgalactiae*, and *Streptococcus uberis*, which can lead to permanent damage of the udder and reduce milk production (Bradley, 2002; Gruet *et al.*, 2001). Contagious mastitis caused by *S. aureus* and *S. agalactiae* was historically the most challenging type of mastitis faced by the industry due to the pathogens' virulence and persistence. However, due to measures taken by producers to control these pathogens, the distribution of the most prevalent pathogens has changed in recent years, and environmental pathogens are now becoming more predominant (Bradley, 2002; Ruegg, 2018; Zadoks and Fitzpatrick, 2009).

Likewise, bovine mastitis can be classified as clinical or subclinical, based on its clinical signs (Fig. 2b). Clinical mastitis is characterized by sudden onset, udder reddening, and swelling (Gruet *et al.*, 2001). Affected quarters produce altered milk, which may have a watery consistency and/or clots and flakes. Cows with clinical mastitis may appear lethargic, have a fever, and poor appetite. The somatic cell count (SCC) in milk is elevated, usually above 300,000 cells/mL (Ruegg, 2017). In contrast, subclinical mastitis is characterized by an absence

of obvious symptoms in milk or the udder, but results in reduced milk production and increased SCC (Halasa *et al.*, 2007; Khan and Khan, 2006; Tancin, 2017). Subclinical mastitis is more prevalent, occurring 15 to 40 times more often than clinical cases and usually lasts longer, making it a pathogen reservoir that spreads infection among animals in the herd (Khan and Khan, 2006; Seegers *et al.*, 2003). Although subclinical cases are not treated until the dry period (Halasa *et al.*, 2007), clinical mastitis treatment is an animal welfare requirement (Medrano-Galarza *et al.*, 2012; Peters *et al.*, 2015). It is usually antibiotic-based, and its effectiveness varies based on the bacterial species present, stage of lactation, type of antimicrobial , route of administration, presence of resistant pathogens, and immune response (Ruegg, 2018, 2021).

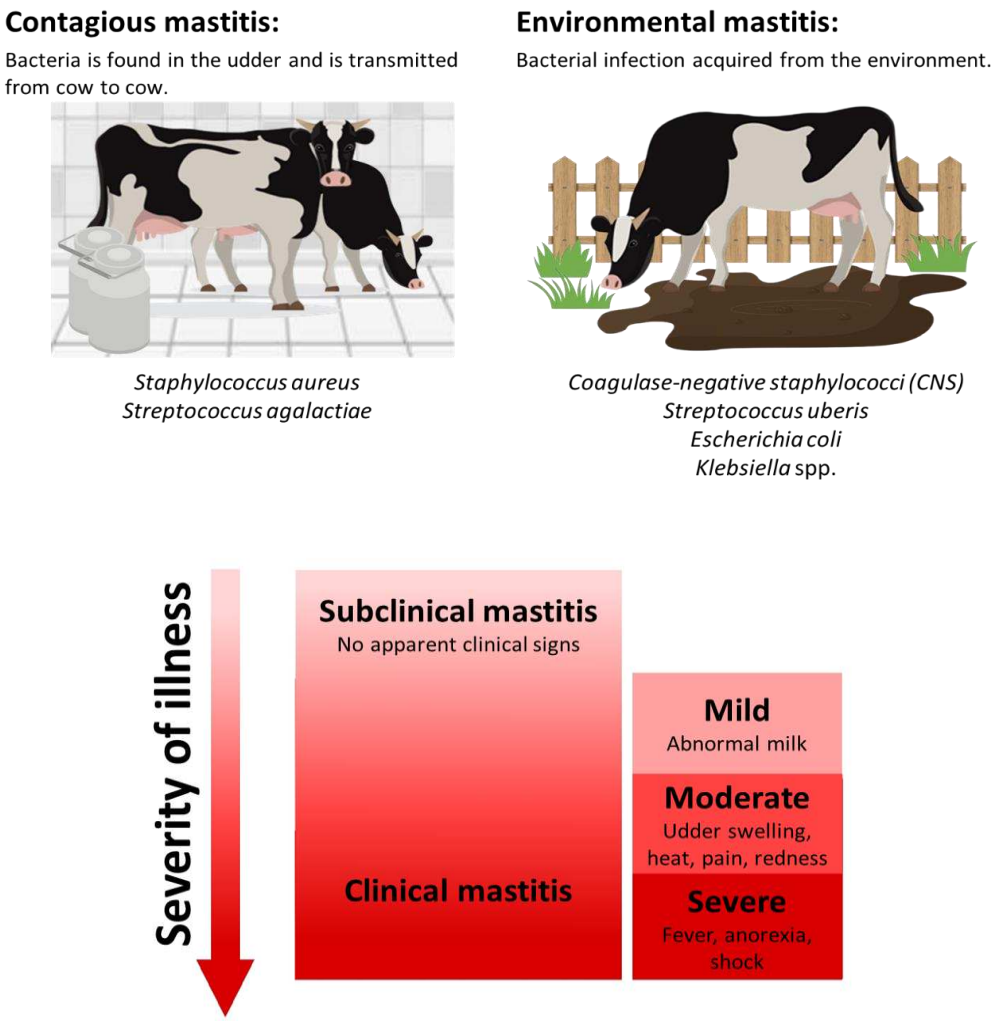

Figure 2: Classification of bovine mastitis according to the origin of pathogen and severity of clinical signs. Source: the author.

MANAGEMENT AND CONTROL STRATEGIES

To reduce the incidence of bovine mastitis in dairy herds, the Five-Point Mastitis Control Plan was created. It is one of the most successful and widely used strategy in the dairy industry to control contagious mastitis. The plan consists of five key components: (1) proper milking procedures, (2) use of post-milking teat disinfectant, (3) dry cow management with the use of antimicrobials, (4) culling of chronically infected cows, and (5) antibiotic treatment of clinical mastitis. These measures aim to prevent the introduction and spread of pathogens, as well as reduce the duration and severity of infections (Kerro Dego, 2021). However, these procedures are not always as effective against environmental pathogens compared to contagious pathogens (Hillerton and Booth, 2018). As a result, in some traditional dairy production regions, such as the United Kingdom ((Peeler *et al.*, 2002), and the United States (Ganda *et al.*, 2016; Oliveira *et al.*, 2013), mastitis prevalence has shifted from contagious to environmental pathogens (e.g., environmental streptococci, *Klebsiella*, *E. coli*) (Ruegg and Petersson-Wolfe, 2018).

One point of the Control Plan is the dry cow therapy, which is the administration of long-acting antimicrobials directly into the udders of dairy cows at dry-off to clear existing intramammary infections and prevent new infections during the dry period, which can otherwise lead to clinical mastitis in the subsequent lactation. Blanket dry cow therapy (BDCT), where all udder quarters of every cow receive long-acting antimicrobial treatment at dry off, has been a longstanding and commonly used practice among dairy farmers (Bradley and Green, 2000, 2004; Green *et al.*, 2002).

The issue with administering antimicrobials to dairy cows as a preventative measure against mastitis is that it places selective pressure on both mastitis-causing bacteria and the commensal bacteria present in the bovine udder. This selective pressure can lead to the development of antimicrobial-resistant bacteria, which are challenging to eradicate and can persist on farms and spread among animals. Ultimately, this resistance may transfer to other bacteria or to humans or animals, posing a broader risk beyond the farm setting (Kerro Dego, 2021). In response to evolving mastitis trends and growing concerns about antimicrobial use in livestock, selective dry cow therapy (SDCT) has emerged as an alternative to BDCT for reducing antimicrobial usage in dairy settings. SDCT involves administering antimicrobial treatment only to cows or quarters suspected of having an infection, while leaving uninfected cows and quarters untreated (Cameron *et al.*, 2014). However, SDCT can only decrease antimicrobial usage at dry off without compromising udder health or milk production in the

early months of the subsequent lactation if internal teat sealants are applied in healthy, untreated quarters or cows (Kabera *et al.*, 2021). Figure 3 illustrates the workflow for each approach.

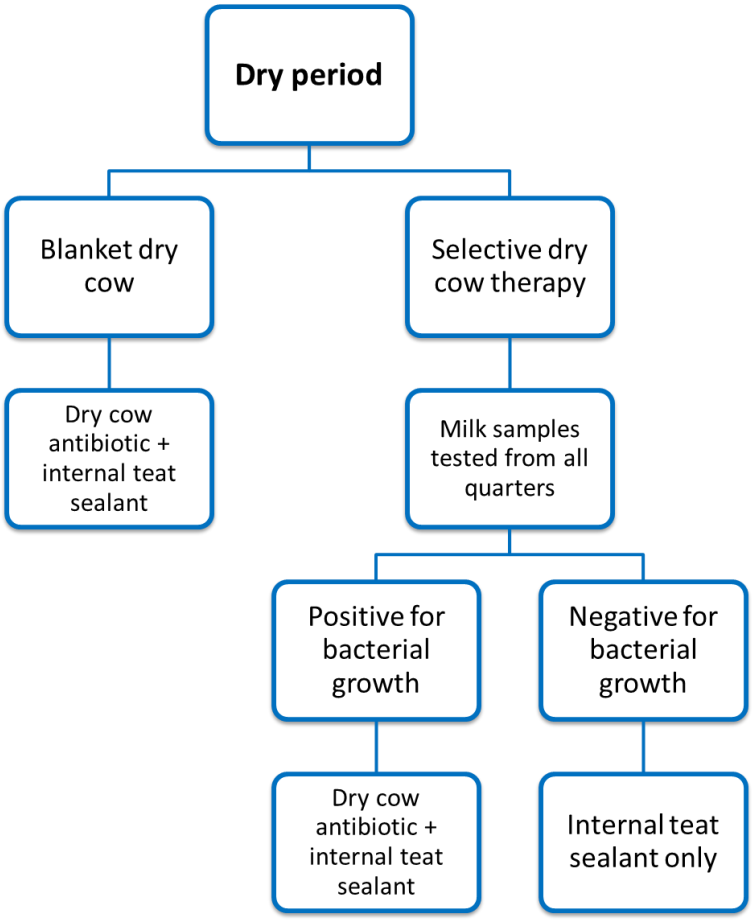

Figure 3: Comparison of decision-making strategies for dry cow therapy: blanket vs. selective approaches. Source: the author.

ANTIMICROBIAL RESISTANCE CONCERNS

The constant use of antibiotics in livestock production systems has led to the emergence of antimicrobial resistance (AMR), which is a significant public health concern under a One Health approach (Rubiola *et al.*, 2020; Ventola, 2015; World Health Organization, 2017). When antibiotics are infused into the mammary glands, they can be released into the environment through milk leakage from the treated udder or absorbed into the bloodstream, metabolized in the liver or kidneys, and then excreted in urine or feces. This process significantly affects the commensal or opportunistic bacteria in the gastrointestinal tract of dairy cows. The widespread use of parenteral and intramammary antibiotics exposes numerous healthy cows to these drugs and amplifies their use on dairy farms, creating intense pressure on

microbes both within the animals' bodies and across the farm environment (Kerro Dego, 2021). Rubiola *et al.* (2020) found AMR genes for eight classes of antibiotics in bulk tank milk samples, including β-lactams, tetracyclines, sulfonamides, fosfomycin, aminoglycosides, multi-drug resistance, rifampin, and macrolide, lincosamide, and streptogramin (MLS) .

As AMR genes can be present in milk and dairy products, they may be introduced into the food chain, making consumers and regulatory agencies increasingly aware of the implications of antibiotic use in animal husbandry (Rubiola *et al.*, 2020; Ventola, 2015; World Health Organization, 2017). Consequently, dairy farms are subject to increasing testing and regulatory control on drug use. To meet the expectations of consumers (Lusk *et al.*, 2006; Rajamanickam *et al.*, 2020) and regulatory agencies such as Infectious Diseases Society of America and the World Health Organization (Bartlett *et al.*, 2013; World Health Organization, 2017), alternatives to antibiotics in livestock production must also be economically viable and not increase the risk of drug residues in marketed milk (Erskine *et al.*, 2003).

ANTIMICROBIAL PEPTIDES AS ALTERNATIVES TO ANTIBIOTICS

To combat AMR, attention is being directed towards bacterial-produced antimicrobial peptides (AMPs) due to their potential chemotherapeutic effect against resistant and biofilm-producing bacterial pathogens (Rouse *et al.*, 2012; Soltani *et al.*, 2021; Zhu *et al.*, 2022). These peptides have high potency, low to no cytotoxicity against eukaryotic cells, and good stability. Resistance against AMPs is rare, as their action targets are usually distinct from those used by antibiotics, reducing the chances of cross-resistance (Yu *et al.*, 2018). Yu *et al.* (2018) evaluated the resistance hazard of a treatment dose by calculating the time-averaged proportion of mutants. This analysis revealed that AMPs are significantly less likely than antibiotics to promote resistance selection at dosages ≤ 1x minimal inhibitory concentration (MIC). In addition, their gene-encoded nature makes them amenable to genetic manipulation and novel drug design (Angelopoulou *et al.*, 2019; Cotter *et al.*, 2013; Mathur *et al.*, 2018; Soltani *et al.*, 2021; Zhu *et al.*, 2022). Some AMPs, such as Nisin, Lacticin 3147, Macedocin ST91KM and Bovicin HC5, have demonstrated the ability to inhibit the growth of mastitis-causing bacteria, including *S. aureus* and *S. agalactiae* in dairy cattle (Barboza-Corona *et al.*, 2009; Godoy-Santos *et al.*, 2019; Pieterse *et al.*, 2010; Roy *et al.*, 2016). Clinical studies have also demonstrated the effectiveness of intramammary administration of n isin to treat contagious mastitis in lactating dairy cows (Cao *et al.*, 2007; Roy *et al.*, 2016). Cao (2007) demonstrated

that nisin Z, when applied intramammarily, showed a clinical cure rate of 90.2%, which was similar to gentamicin.

The rumen microbiome remains a largely unexplored reservoir for the identification of novel microbial enzymes and metabolites, including antimicrobial peptides (AMPs) (Oyama *et al.*, 2017). Within this dynamic and competitive microbial community inhabiting the ruminal environment, lies a promising opportunity for the discovery of new AMPs. Lynronne-1 and Lynronne-2, linear antimicrobial peptides (AMPs), were identified through functional screening of the rumen bacterial metagenome and computational methods (Oyama *et al.*, 2017). These peptides possess several characteristics that render them promising candidates for mastitis therapy, including potent activity against key mastitis-causing pathogens, favorable kinetics against biofilms, and low toxicity. Both peptides demonstrate effectiveness against a wide range of Gram-positive pathogens. When tested against *S. aureus* biofilms, Lynronne-1 and Lynronne-2 exhibited the ability to reduce biofilm attachment at concentrations twice the minimum inhibitory concentration (MIC), while also displaying anti-biofilm activity against established biofilms at concentrations equal to or greater than 2× MIC. Additionally, these AMPs demonstrated rapid eradication of *E. coli* K_{12}, with complete cell death occurring within 10 minutes at concentrations three times the MIC. Importantly, Lynronne-1 and Lynronne-2 showed minimal hemolytic activity against blood cells and negligible cytotoxicity against mammalian cells. Furthermore, prolonged exposure to sub-lethal concentrations of Lynronne-1 and Lynronne-2 did not result in decreased susceptibility of m ethicillin-resistant *Staphylococcus aureus* (MRSA), and no mutants resistant to the AMPs were isolated even after 25 days. These findings highlight the potential of Lynronne-1 and Lynronne-2 as safe and effective alternatives for combating mastitis and other bacterial infections (Mulkern *et al.*, 2022; Oyama *et al.*, 2017).

Bovicin HC5 is another important AMP discovered among ruminal bacteria . Produced by *Streptococcus bovis* HC5, bovicin HC5 is classified as a broad-spectrum bacteriocin. It exhibits a spectrum of activity akin to monensin, a commonly used feed additive in animal rations. Bovicin HC5 demonstrates inhibitory effects against various Gram-positive bacteria, including species of streptococci, lactococci, clostridia, enterococci, bacilli, and lactobacilli (Houlihan and Russell, 2006; Mantovani *et al.*, 2002). Preliminary investigations have revealed that bovicin HC5 exhibits promising capabilities in controlling the growth of bacterial pathogens and impeding bacterial adhesion and biofilm formation (Mantovani and Russell, 2003; Pimentel-Filho *et al.*, 2014). Moreover, studies have indicated low enteral

toxicity of bovicin HC5 *in vivo* (Paiva *et al.*, 2012), suggesting its potential suitability for application in the food industry (de Carvalho *et al.*, 2006, 2007, 2008) and as an alternative to traditional antibiotics in livestock production (Godoy-Santos *et al.*, 2019). Of particular significance is the heightened sensitivity of *S. aureus*, the predominant and persistent pathogen causing mastitis, to bovicin HC5. Furthermore, coagulase-negative staphylococcal strains, often associated with mild udder inflammation and reduced milk production, also display high susceptibility to bovicin HC5. Notably, bovicin HC5 exhibits robust inhibitory activity against mixed cultures of staphylococci and streptococci (Godoy-Santos *et al.*, 2019). Still, only Gram-positive strains were affected by bovicin HC5 alone, with *Escherichia coli* strains showing no susceptibility. However, when combined with Ethylenediaminetetraacetic acid (EDTA), inhibitory activity can be achieved against *Salmonella enterica* serovar Typhimurium, probably due to the chelating activity of EDTA, which affects the integrity of the outer membrane of G ram- negative bacteria (Prudêncio *et al.*, 2014; Prudêncio *et al.*, 2016).

While the gastrointestinal tract of ruminants has been a fruitful environment for the discovery of antimicrobials, it is important to recognize that novel antimicrobials can emerge from various sources (Hancock and Sahl, 2006; Maher and McClean, 2006; Wolfgang *et al.*, 2016). Utilizing artificial intelligence methodologies such as natural language processing techniques in combination with neural network models has led to the identification of AMP660 and AMP1043 from the human gut microbiome. These peptides have demonstrated promising *in vitro* efficacy against a wide range of multidrug-resistant (MDR) bacterial strains, including *E. coli*, *Acinetobacter baumannii*, *K. pneumoniae*, *S. aureus*, and *Bacillus subtilis*, with minimum inhibitory concentrations (MICs) below 50 μM. Moreover, both AMPs exhibit relatively low cytotoxicity and hemolytic activity, with AMP660 displaying a slightly higher, yet still negligible, hemolysis rate. AMP1043 has shown the ability to disrupt the outer membrane potential of Gram-negative bacteria, thereby contributing to their inhibition, with a time-to-kill observed within 2-6 hours. Furthermore, *in vivo* assays demonstrated the capacity of AMP1043 to control *K. pneumoniae* infection in mice, resulting in a significant reduction of bacterial load by over tenfold. Importantly, no resistance was observed in *E. coli* cultures even after 30 days of treatment. These findings highlight the potential of AMP660 and AMP1043 as promising candidates for combating multidrug-resistant bacterial infections (Ma *et al.*, 2022).

A NEW STRATEGY AGAINST RESISTANT MICROORGANISMS

One effective strategy to combat AMR involves combining different antimicrobials. By combining two or more antimicrobials, especially those targeting different pathways, it is possible to achieve superior efficacy against resistant pathogens. Benefits of combination therapy include reduction of dosage and associated toxicity, and suppression of emerging resistance (Allan, 1987; Petrosillo *et al.*, 2013; Ventola, 2015; Zimmermann *et al.*, 2007). Because monotherapy options all have significant limitations (pharmacokinetics, toxicity, emergence of resistance), combination therapy can be an attractive option to optimize therapy (Morrill *et al.*, 2015).

For example, in four distinct clinical trials, antibiotic combination therapy proved more effective than monotherapy for treating Carbapenem-Resistant Enterobacteriaceae bloodstream infections, resulting in a significant reduction in mortality rates ($P<0.05$). Bloodstream infections caused by Carbapenemase-producing *Klebsiella pneumoniae* are associated with high mortality. Despite patients receiving appropriate antimicrobial monotherapy based on *in vitro* susceptibility, high mortality rates persisted across all four trials (Zarkotou *et al.*, 2011; Qureshi *et al.*, 2012; Tumbarello *et al.*, 2012; Daikos *et al.*, 2014). Another example of combinatorial therapy is pairing a beta-lactam antibiotic with a beta-lactamase inhibitor. This combination has been extensively researched and applied to combat beta-lactam resistance in Gram-negative bacteria such as *E. coli* and *K. pneumoniae* (Bush *et al.*, 2011). These synergistic interactions can disrupt resistance mechanisms such as enzymatic degradation or efflux pumps, thereby restoring the therapeutic potential of existing antibiotics (Bush *et al.*, 2011). Moreover, this approach underscores the significance of innovative strategies in the ongoing battle against antibiotic resistance, highlighting the potential of combination therapies to address this critical healthcare challenge.

In the case of eukaryotic innate immune system AMPs, synergy occurs naturally when these molecules are expressed together in animal tissues, as demonstrated by previous studies (Lai and Gallo, 2009; Zhu *et al.*, 2022). The synergistic effect of AMPs is believed to be effective in countering various resistance mechanisms used by pathogenic bacteria (Guilhelmelli *et al.*, 2013; Zhu *et al.*, 2022). Numerous studies have demonstrated a synergistic effect between AMPs and antibiotics when combined (Ellis *et al.*, 2019; Kim *et al.*, 2017; Pletzer *et al.*, 2018; Song *et al.*, 2020.; Thappeta *et al.*, 2020; Wu *et al.*, 2021; Zhu *et al.*, 2022). Thappeta *et al.* (2020) revealed that the combination of AMP CSM5-K5 with conventional antibiotics restored drug sensitivity against multidrug-resistant clinical isolate *Enterococcus faecalis* V583, which is resistant to both oxacillin and vancomycin. The MIC values were

reduced significantly after combination, with the MIC of CSM5-K5 reduced to 0.25× MIC, and the MIC of vancomycin and oxacillin reduced by 8-fold and 16-fold, respectively. AMPs may also exhibit a synergistic interaction with non-antibiotic antimicrobial compounds. Noll *et al.* (2012) combined *Bacillus amyloliquefaciens*-produced AMP subtilosin with various non-antibiotic antimicrobials to inhibit human pathogen *Gardnerella vaginalis* growth. The MIC of subtilosin was reduced by 2-fold, while glycerol monolaurate and lauric arginate had a 4-fold reduction in their MIC, and polylysine exhibited a significant 10-fold reduction. Therefore, combined use of AMPs has the potential to increase their effectiveness.

In conclusion, while the challenges posed by bovine mastitis are multifaceted, the emergence of AMPs as promising alternatives to conventional antibiotics offers renewed hope for sustainable mastitis control strategies that prioritize animal welfare, food safety, and public health. By harnessing the potential of AMPs and leveraging synergistic interactions with existing antimicrobial compounds, the dairy industry can strive towards a future characterized by reduced disease burden, improved productivity, and enhanced sustainability.

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CHAPTER 1

ANTIMICROBIAL PEPTIDE-BASED COMBINATIONS SHOW ACTIVITY AGAINST PATHOGENIC BACTERIA AFFILIATED WITH BOVINE MASTITIS

ABSTRACT

Bovine mastitis is the costliest disease in the dairy sector and is the main cause of antibiotic use in dairy cattle, potentially contributing to the antimicrobial resistance problem. Antimicrobial peptides (AMPs) have been proposed as alternatives to antibiotics to control bovine mastitis pathogens. In this study, the efficacy of five AMPs (Lynronne-1 (Lyn-1), Lynronne-2 (Lyn-2), Bovicin HC5, AMP 660 and AMP 1043) and two bioactive compounds (disodium ethylenediaminetetraacetic acid – EDTA and glycerol monolaurate - GML) was assessed against a range of 35 mastitis-causing pathogens, including *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcus chromogenes, Streptococcus agalactiae, Streptococcus uberis,* and *Streptococcus dysgalactiae* strains. Lyn-1 and EDTA were the most effective treatments exhibiting the highest broad spectrum antimicrobial activity and reducing the final bacterial optical density (OD_{600} nm) by 95.1% and 86.9%, respectively. In combinatorial assays assessed by the Fractional Inhibitory Concentrations Index (FICI) to determine the interaction and synergistic effect of the compounds, FICI values ranged from 0.1 to 0.5, indicating synergism. The combination of lyn-1 (32 µg/mL) and EDTA (1.0 g/L) exhibited higher antimicrobial activity against all bacterial strains, at significantly lower concentrations (4 and 8-fold reduction for lyn-1 and EDTA, respectively) than each compound individually. Lyn-1-EDTA combination was bactericidal, reducing the viable population by 1,0000-fold (4.55 log-cycles) within 12 hours. Moreover, the combination was non-hemolytic in concentrations up to 8-fold the established MIC values ($P > 0.05$), although cytotoxic effects assessed by MTT assay were observed at concentrations above MIC ($P < 0.01$) in epithelial bovine mammary alveolar (MAC-T) cells treated with the lyn-1-EDTA combination. These results provide pivotal evidence that lyn-1 and the lyn-1-EDTA combination have therapeutic potential and can be used as a basis for developing antibiotic-free formulations to control contagious and environmental mastitis pathogens and in the treatment of udder infections.

Key words: bovine mastitis, antimicrobial peptides, synergism, combinatorial therapy.

INTRODUCTION

Mastitis is an inflammatory disease of the mammary gland typically caused by contagious or environmental microorganisms, mainly bacteria. Bovine mastitis is considered the costliest disease in the dairy sector and can significantly affect the production performance of lactating cows and profitability (Oliveira, Hulland and Ruegg, 2013; Rollin, Dhuyvetter and Overton, 2015; Ashraf *et al.*, 2018; Ruegg, 2020). A retrospective observational study based on data collected from 37 Wisconsin dairy herds indicated that the average cost of intramammary therapy and milk discard associated with clinical mastitis was $192.36 ± 8.90 per case and overall direct costs were highly influenced by the duration of therapy (de Campos *et al.*, 2023) In the United States, bovine mastitis is estimated to cost approximately $2 billion annually to the dairy industry (Cobirka, Tancin and Slama, 2020).

Treatment of clinical mastitis is considered an animal welfare requirement (Medrano-Galarza *et al.*, 2012; Peters, Silveira and Fischer, 2015) and even non-severe cases are typically treated with antibiotics. However, the prophylactic and therapeutic use of antibiotics in livestock production systems, including dairy farms, has been linked to the emergence of antimicrobial resistance, which is a significant public health concern (Ventola, 2015; World Health Organization, 2017; Rubiola *et al.*, 2020). Moreover, the detection of antimicrobial resistance genes in food products of animal origin such as milk reduces the market value of these products and emphasizes the need for new and effective approaches to prevent the development, persistence, and spread of resistant bacteria.

A promising alternative to reduce the use of antibiotics in dairy farms to combat resistant and biofilm-producing bacterial pathogens, such as those associated with bovine mastitis, is to develop combinatorial therapies based on antimicrobial peptides (AMPs) (Rouse *et al.*, 2012; Mathur *et al.*, 2018; Soltani *et al.*, 2021; Zhu *et al.*, 2022). AMPs have high potency, low cytotoxicity to eukaryotic cells, and good stability over a broad range of temperatures and pH values (Cotter, Ross and Hill, 2013; Soltani *et al.*, 2021). Resistance against AMPs is considered rare, as their distinct mechanisms of action reduce the chances of cross-resistance to conventional antibiotics (Yu *et al.*, 2018; Soltani *et al.*, 2021).

The rationale behind the use of two or more bioactive molecules to control microbial infections is that a combination of different antimicrobials broadens and enhances their killing efficacy against pathogens, thus providing an effective and safe alternative to conventional

antibiotic-based treatment while reducing the selection of resistance (Zimmermann, Lehár and Keith, 2007). Additionally, the combination of antimicrobials that act on different pathways or molecular targets, can help reduce therapeutic dosage and associated toxicity of bioactive compounds (C. Lee Ventola, 2015). In the case of the eukaryotic innate immune system, synergy between AMPs can occur naturally when different host defense peptides are expressed in animal tissues and may help prevent colonization by pathogenic bacteria (Lai and Gallo, 2009; Guilhelmelli *et al.*, 2013; Zhu *et al.*, 2022). Moreover, numerous studies have demonstrated a synergistic effect when antibiotics are combined with AMPs (Kim, Rajasekaran and Shin, 2017; Pletzer, Mansour and Hancock, 2018; Thappeta *et al.*, 2020; Wu *et al.*, 2021; Zhu *et al.*, 2022). AMPs may also exhibit a synergistic interaction with non-antibiotic antimicrobial compounds. Noll *et al.* (2012) combined subtilosin produced by *Bacillus amyloliquefaciens* with various non-antibiotic antimicrobials and evaluated their efficacy in inhibiting the growth of the human pathogen *Gardnerella vaginalis*. The MIC of subtilosin was reduced 2-fold in the combination, while the other antimicrobials (glycerol monolaurate, lauric arginate, and polylysine) exhibited a 4 to 10-fold reduction in MIC. These results indicate that combining AMPs with other non-antibiotic antimicrobial compounds can potentially increase their effectiveness against bacterial pathogens.

Some AMPs such as nisin, lacticin 3147, macedocin ST91KM, and bovicin HC5 can inhibit the growth of mastitis-causing bacteria, including *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from dairy cattle (Barboza-Corona *et al.*, 2009; Pieterse, Todorov and Dicks, 2010; Roy, Riley and Crabb, 2016; Godoy-Santos *et al.*, 2019). However, studies on the synergistic interactions of AMPs with non-antibiotic antimicrobials against mastitis-causing pathogens are limited. Moreover, the rumen and human gut microbiome have been identified as underexplored resources of novel microbial enzymes and metabolites, including AMPs (Oyama *et al.*, 2017, 2022; Ma *et al.*, 2022). Here, we aimed, for the first time, to investigate the efficacy of AMPs derived from the rumen and human gut microbiomes against a range of mastitis bacterial pathogens and assess potential synergistic interactions with non-antibiotic molecules. Four synthetic AMPs (lynronne-1, lynronne-2, AMP660, and AMP1043) (Oyama *et al.*, 2017; Ma *et al.*, 2022) and one naturally produced peptide (bovicin HC5) (Mantovani *et al.*, 2002) were selected based on their potency against a range of bacterial pathogens, including species associated with bovine mastitis. Lynronne-1 and lynronne-2 are linear antimicrobial peptides (AMPs) discovered through functional screening of the rumen bacterial metagenome and computational approaches (Oyama *et al.*, 2017; Ma *et al.*, 2022).

AMP660 and AMP1043 were discovered in the human gut microbiome using natural language processing methods combined with neural network models (Ma *et al.*, 2022). Bovicin HC5 is a broad-spectrum ruminal bacteriocin produced by *Streptococcus equinus* HC5 with low cytotoxicity against eukaryotic cells (Mantovani *et al.*, 2002). We hypothesized that AMPs would have high killing efficacy against mastitis pathogens and their bactericidal effect could be enhanced when combined with other non-antibiotic antimicrobials.

MATERIAL AND METHODS

Bacterial strains and culture conditions

A panel of 35 strains representing different species of major mastitis pathogens including Gram-positive (n = 25) and Gram-negative (n = 10) bacteria were tested as target organisms in the current study. The target bacteria included contagious and environmental Staphylococci and Streptococci, *Escherichia coli*, and *Klebsiella pneumoniae*. The strains were isolated from milk samples collected from cows with signs of clinical mastitis and submitted to the Wisconsin Veterinary Diagnostic Laboratory (WVDL) for routine diagnostics by an accredited veterinarian. Milk samples were taken from individual animals and cultured on sheep blood agar (Becton Dickinson, Franklin Lake, NJ), Eosin Methylene Blue (EMB) agar (prepared in-house), Modified Edwards agar (Udder Health Systems Inc., Meridian, ID) as described by the National Mastitis Council Handbook (National Mastitis Council, 2017) and approved by the American Association of Veterinary Laboratory Diagnosticians. All plates were incubated at 36ºC with 5% CO_2 and examined for bacterial growth at 24 and 48 hours. Predominant organisms were considered pathogens and were further identified.

Pathogens were identified using matrix-assisted laser desorption ionization time-of-flight mass spectroscopy Biotyper 3.1 (MALDI-TOF MS; Bruker Daltonik; Bremen, Germany) and/or using biochemical assays as described by the NMC Handbook (National Mastitis Council, 2017). Bacterial colonies were plated in duplicate onto MALDI-TOF MS polished steel plates. The direct method using formic acid overlay was performed to extract proteins from the bacteria as described by the manufacturer. Isolates obtaining a score from 2.3 to 3.0 were reported to the genus and species level. For this study, only those isolates that were identified to the genus and species level from 2020-2022 were used for further analysis.

Antimicrobial agents

The AMPs tested in this study against mastitis pathogens included lynronne-1 (lyn-1) and lynronne-2 (lyn-2) (Oyama *et al.*, 2017; Adam J. Mulkern *et al.*, 2022), bovicin HC5 (HC5) (Mantovani *et al.*, 2002; Paiva *et al.*, 2012; Godoy-Santos *et al.*, 2019), AMP 660 and AMP 1043 (Ma *et al.*, 2022). Lyn-1, lyn-2, and HC5 originated from the rumen microbiome while AMP 660 and AMP 1043 were identified in the human gut microbiome. The peptides were selected based on i) reported efficacy against bacterial species associated with mastitis infections, ii) spectrum of action, and iii) safety against eukaryotic cells. Bovicin HC5 was produced and purified in the laboratory. Briefly, cells from stationary phase *Streptococcus equinus* HC5 culture were harvested by centrifugation. The extraction was performed by incubating the cells in acidic sodium chloride (100 mM, pH 2·0) overnight at 4 °C. The cells were removed by centrifugation and the cell-free supernatants were purified using a solid-phase extraction column (Sep-Pak® C18 Plus Short Cartridges). The column was equilibrated with 10 mL of a 0.1% formic acid aqueous solution. 50 mL extract was injected and eluted using 5 mL of a 50% acetonitrile aqueous solution and 0.1% formic acid, at 22°C recovering the first 3 mL, which contained bovicin HC5, and the rest was discarded. The recovery of antibacterial activity of the eluted fractions was assayed by agar well-diffusion assays (Devillers *et al.*, 1989) using *Lactococcus lactis* ATCC 19435 (10^5 CFU/mL) as the target organism. The purified bovicin HC5 was lyophilized and stored at −20°C until further use. Lyn-1, lyn-2, AMP 1043, and AMP 660 were chemically synthesized by Peptide 2.0 Inc. (Chantilly, USA) and resuspended in sterile ultrapure water.

In addition, disodium ethylenediaminetetraacetic acid (EDTA) (Thermo Fischer Scientific, Waltham, EUA) and Glycerol monolaurate (GML) (Natural Biologics LLC, Albert Lea, EUA) were also selected to be tested in this study alongside the AMPs. Disodium EDTA and GML are additives commonly used in foods for human consumption and generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (U.S. Food and Drug Administration, 2019). These compounds have proven antimicrobial activity and previous studies indicated potential synergistic effects when combined with bioactive peptides (Schlievert and Peterson, 2012; Prudencio, Mantovani and Vanetti, 2014; Khan *et al.*, 2015). The stock solution of EDTA was prepared in ultra-pure water, while GML stocks were prepared in 100% ethanol.

Determination of MIC values of the antimicrobial agents

The minimal inhibitory concentrations (MICs) were determined using standard broth microdilution susceptibility testing method in sterile 96-well polypropylene plates (Wiegand, Hilpert and Hancock, 2008). In brief, twofold serial dilutions of the AMPs were added to individual wells in each row of the plate. The optical density of the bacterial target was adjusted to an OD_{600} of ~0.08 in cation-adjusted Mueller–Hinton broth (Millipore Sigma, Burlington, EUA) using a Genesys30® spectrophotometer (Thermo Fischer Scientific, Madison, EUA). Next, the culture was diluted 100-fold to obtain a final concentration of 5×10^5 CFU/mL in each well of the microtiter plates containing MH broth (100 µL). The plates were incubated at 37°C for 24 h. Bacterial growth was determined by measuring absorbance at 600 nm in a microplate spectrophotometer SpectraMax Plus 384® (Molecular Devices, LLC., San Jose, USA). The MIC was expressed as the lowest concentration of antimicrobial without an increase in optical density (Wiegand *et al.*, 2008). The highest concentration of antimicrobials tested was 128 mg/L for each AMP, 2.7 g/L for disodium EDTA, and 2.2 g/L for GML. For reference, the MIC assays were also performed with ceftiofur (CEF), a cephalosporin-antibiotic commonly used for intramammary infusions in U.S. dairy farms to treat udder infections (Durel, Gallina and Pellet, 2019; Rajamanickam *et al.*, 2020). All assays were conducted in triplicate and with two biological replicates.

Checkerboard synergy assays

Synergy assays were performed using the broth microdilution checkerboard method to evaluate interactive inhibition between antimicrobials (Hsieh *et al.*, 1993; Fratini *et al.*, 2017; Cokol-Cakmak and Cokol, 2019). Assays were carried out on 96-well microtiter plates based on MIC values determined as described above. The two antimicrobials (lyn-1 and EDTA) showing the highest activity against a broad range of mastitis pathogens were serially diluted two-fold at a starting concentration of 128 mg/L. The lyn-1 dilutions were distributed (20 µL) across each row (x-axis) of the 96-well plate, while the EDTA dilutions were dispensed (20 µL) across columns (y-axis) to obtain final concentrations equivalent to MIC, MIC1/2, MIC1/4, and MIC1/8, MIC1/16, and MIC1/32 of each antimicrobial. The volume in each well was then completed to 100 µL with sterile cation-adjusted MH media. Next, a cell suspension containing equivalent amounts (standardized at 5×10^5 $CFUmL^{-1}$) of five strains of each bacterial species tested in the current study was added (100 µL) into wells on a microtiter plate. For the negative control, each well was completed with 100 µL of sterile Mueller–Hinton broth. Microplates

were incubated at 37ºC for 24 h. Bacterial growth was determined by measuring the optical density (OD_{600}), and the results were normalized according to the equation:

$$O.D._{COMBINATION} - O.D._{BLANK} / O.D._{POSITIVE\ CONTROL} - O.D._{BLANK}$$

Negative controls (no inoculum) were set to 0 and the positive controls (growth control, no inhibitors) were set to 1 (100 %). Treatments were represented with a value between 0 and 1, indicating growth percentage. Formulations with an $OD_{600} \leq 0.1$ (or 90 % of inhibition) were selected for Fractional Inhibitory Concentrations Index (FICI) determination. The FICI is a quantitative measure of the combination effect, where lower values indicate a stronger synergistic interaction between bioactive compounds. The combination showing the smallest FICI value was selected in the current study for further analysis. Fractional Inhibitory Concentration (FIC) determinations were performed with two biological and two technical replicates. For each replicate, FICI values were calculated as follows:

$$FICI = FIC_{LYN\text{-}1} + FIC_{EDTA}$$

where $FIC_{LYN\text{-}1} = MIC_{LYN\text{-}1}$ in combination/$MIC_{LYN\text{-}1}$ alone and $FIC_{EDTA} = MIC_{EDTA}$ in combination / MIC_{EDTA} alone (EUCAST, 2000).

FICI values designate the interaction effect between antimicrobials. A FICI value of ≤0.5 represented synergism, a FICI value between 0.5 and 4.0 represented indifference, and a FICI > 4.0 represented antagonism (Odds, 2003).

Time-dependent killing assays

The time-dependent killing assays were performed in Mueller Hinton broth with the antimicrobials selected above using the MIC values defined for each compound in isolation and the dose-reduced concentration of the compounds combined at 1x and 2x MIC. The inoculum was adjusted for an initial cell density of approximately 10^5 CFU/mL. The strains of *K. pneumoniae* and *S. aureus* that showed the highest MIC values for lyn-1 were selected as targets for this experiment. The treated cultures were incubated at 37°C and samples (10 µL) were taken at different time intervals (0, 11, 22, 45, 90, 180, 360, 720, and 1440 min). After serial 10-fold dilutions in phosphate-buffered saline (PBS), the samples (5 µL) were plated on a BHI agar plate by the agar droplet technique (Sharpe and Kilsby, 1971). Cell viability was estimated after 12 hours of incubation using the colony counting method. The bacterial death kinetics was represented by plotting viable cell counts against time (Ojo and Ejims-Enukwe, 2013). Controls

consisted of cultures treated with lyn-1 and EDTA alone at MIC values and cultures not exposed to the antimicrobials. This assay was conducted in duplicate and with three biological replicates.

Image processing and analysis

Samples collected from different time intervals during the time-kill assay were spotted on BHI agar plates and incubated for 12 hours at 37°C. Following incubation, the resulting bacterial mass generated in each spot was photographed and the image area was determined by pixel-based assessment. Initially, image enhancement was performed using the Clone Stamp Tool within Photofiltre Studio X 10.14.1 software (da Cruz, 2020). This step aimed to eliminate any potential interference from petri dish edges that could impact subsequent pixel analysis. Afterwards, the images were processed using ImageJ software (Schneider, Rasband and Eliceiri, 2012). First, images were converted to an 8-bit format, and pixel measurements were refined to impose both a limit and a specific threshold. Next, the aggregate pixel count of each bacterial biomass spot was determined. The acquired data were then subjected to normalization. For each time point, normalization was relative to the untreated control and the initial time point, which were standardized at 100%. This experimental protocol was executed with two biological replicates.

Determination of hemolytic activity

Hemolytic activity of the antimicrobial combination was determined by the colorimetric hemoglobin release assay. Fresh bovine erythrocytes were washed with PBS buffer (pH 7.0), diluted 36x in PBS buffer, and plated in 96-well flat-bottomed polystyrene plates. The antimicrobial combination was added to the wells (20 µL), along with lyn-1 and EDTA alone, at 1x, 2x, 4x, and 8 x MIC. The plates were incubated for 3 h at 37°C, centrifuged and aliquots of the supernatant were transferred to a fresh 96-well plate to determine the release of hemoglobin at 543 nm in the microplate reader. A solution of 0.1% Triton X was used as the control and wells containing PBS solution were used as blanks (Oyama *et al.*, 2017). Normalization for comparison was conducted by assigning 100% to the average absorbance of the Triton X control. Assays were conducted with two biological replicates, each consisting of four technical replicates.

Epithelial bovine mammary alveolar cells and culture conditions

Epithelial bovine mammary alveolar (MAC-T) cells were cultured in T75 cell culture flasks with DMEM medium (Sigma-Aldrich, San Luis, EUA) containing 10% heat-inactivated fetal calf serum (Sigma-Aldrich, San Luis, EUA), 100 U/mL penicillin, 10 g/L streptomycin (Sigma-Aldrich, San Luis, EUA), and 5 g/mL insulin (Sigma-Aldrich, San Luis, EUA). Cells were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2. Cells were cultured to reach a confluent monolayer by visual inspection using a light microscope. The MAC-T cells were then treated with 0.05% trypsin (Sigma-Aldrich, San Luis, EUA) (Sipos and Merkel, 1970), and resuspended in fresh MAC-T medium at a concentration of 10^5 cells/mL. For cytotoxicity assays, MAC-T cells were seeded in 96-well tissue culture treated plates (10^5 cells/well) and incubated overnight at 37°C under 5% CO_2 to obtain a confluent monolayer.

Safety analysis of the lyn-1-EDTA combination against epithelial mammary alveolar cells

The effect of the antimicrobial combination on the viability of MAC-T cells was evaluated by a quantitative colorimetric assay based on tetrazolium salt. In short, the MAC-T cell line was cultured in a 96-well plate containing DMEM medium at an initial density of 1×10^5 cells per well. The culture was incubated overnight at 37°C under an atmosphere of 5% CO_2 to allow attachment and formation of monolayers. A volume of 3 mL of working solutions was added, which contained the antimicrobial combination, as well as lyn-1 and EDTA alone, at 1x, 2x, 4x, and 8 x FIC diluted in DMEM 5% heat-inactivated fetal calf serum and 5 g/mL insulin. If a decrease in pH was observed, a sterile solution of NaOH 10 M was added to turn the color of the medium to red (pH 7.2). After 16 h incubation, the supernatants were removed and the MTT Reagent® (R&D Systems, Minneapolis, USA) was added to each well at a proportion of 10 µL per 100 µL of the medium. The plates were incubated for 2 h at 37°C and the dark blue formazan crystals formed were dissolved by adding 100 µL of DMSO (dimethyl sulfoxide) to each well followed by quick mixing. Absorbance at 590 nm was determined after 15 min with a microplate reader. Non-treated cells, added to fresh DMEM, were used as a positive control. Cells treated with 0.1% Triton-X were used as the negative control. The percentage of viable cells was calculated based on the ratio between the absorbance of treated and control cells (At/Ac×100) (Mosmann, 1983; Maher and McClean, 2006; Paiva *et al.*, 2012). The integrity of the cell lines was also observed on an inverted optical microscope during the assays to determine the effects on cell morphology as loss of monolayer, granulation, and vacuolization of the cytoplasm. The assays were conducted with two biological replicates, each consisting of three technical replicates.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8.4.3 software (Harvey Motulsky, 2023). Data normality was assessed using the Shapiro-Wilk test. Differences were considered significant when $P < 0.05$. For minimal inhibitory concentration assays, non-parametric data sets were analyzed using the Kruskal-Wallis test, followed by post-hoc Dunn's test for pairwise comparisons. Parametric data sets were subjected to a one-way ANOVA, followed by Dunnett's test. To determine statistical significance between concentrations, the final OD_{600} for each antimicrobial concentration was compared to that of the untreated control. Furthermore, the final OD_{600} of each treatment at its highest tested concentration was compared to that of the untreated control to assess statistical differences between treatments. The inhibition percentage for each strain was calculated, and significant differences between treatments and the control were assessed using the Multiple Mann-Whitney test. Additionally, the inhibition percentage for each treatment was computed.

To compare FICI values between species, a one-way ANOVA was employed to assess significant differences. For analysis of time-dependent killing and pixel data, a Two-way ANOVA was performed. The area under the time-kill curve (AUC) of each treatment was calculated and statistical differences were assessed by a one-way ANOVA followed by Tukey's test. The hemolysis and cytotoxicity results were evaluated using the Kruskal-Wallis test to assess significant differences between treatments, followed by post-hoc Dunn's test for pairwise comparisons.

RESULTS

Activity of Antimicrobial Peptides, EDTA, and Glycerol Monolaurate

When AMPs, EDTA, and GML were tested against a range of mastitis pathogens, significant differences in inhibition ($P < 0.05$) were observed for all antimicrobials against particular strains of mastitis pathogens or different species of bacteria tested in the current study (Fig. 1a). However, the spectrum of activity was markedly distinct among inhibitors, with some molecules showing broad activity against both Gram-positive and Gram-negative bacteria while others effectively targeted only a limited number of species or specific strains within a species (Fig. 1a). Lyn-1 and EDTA showed broader spectrum of activity (Fig. 1a) and greater

killing efficacy (Fig. 1b), reducing the final OD of all 35 target strains by 95.1% and 86.9%, respectively (Fig. 1b). Lyn-2 was primarily effective against Gram-negative bacteria (81.1% reduction in mean OD_{600}) and the environmental Streptococci, but had little activity against *S. aureus* (Fig 1). AMP 660 showed broad antimicrobial activity, but the efficacy was lower compared to lyn-1 and lyn-2 with approximately 44% reduction in the mean OD_{600}. Bovicin HC5 was effective against the streptococci associated with bovine mastitis, but showed limited activity against the Gram-negative bacteria and *S. aureus* strains (Fig. 1). AMP 1043 was the least effective of the AMPs and only caused an 11.4% reduction in the OD_{600} of the treated cultures (Fig. 1b). The spectrum of activity of GML was similar to AMP 660, and the average inhibition of bacterial growth (OD_{600}) was 43.8% (Fig. 1b).

The three antimicrobials showing a broader spectrum of activity and greater efficacy against mastitis pathogens (lyn-1, lyn-2, and EDTA) were selected for the checkerboard synergy assays. Initially, lyn-1, lyn-2, and EDTA were subjected to orthogonal dilution (lyn-1 and lyn-2, 128 mg/L to 4 mg/L; EDTA, 2,689 mg/L to 21 mg/L), and the effect on bacterial growth was evaluated spectrophotometrically (Fig. 2). Results confirmed the higher antimicrobial activity of lyn-1 and EDTA against the tested bacteria, with MIC ranging from 8 to 128 mg/L for lyn-1 and 672 mg/L to >2,689 mg/L for EDTA (Supplementary Table 1). MIC values were ascertainable for only 37.14% (n = 13) of the bacteria treated with lyn-2, with the remainder being deemed >0.12 mg/mL. In particular, *E. coli* strains exhibited higher susceptibility to this AMP whereas *S. aureus* strains demonstrated the lowest susceptibility.

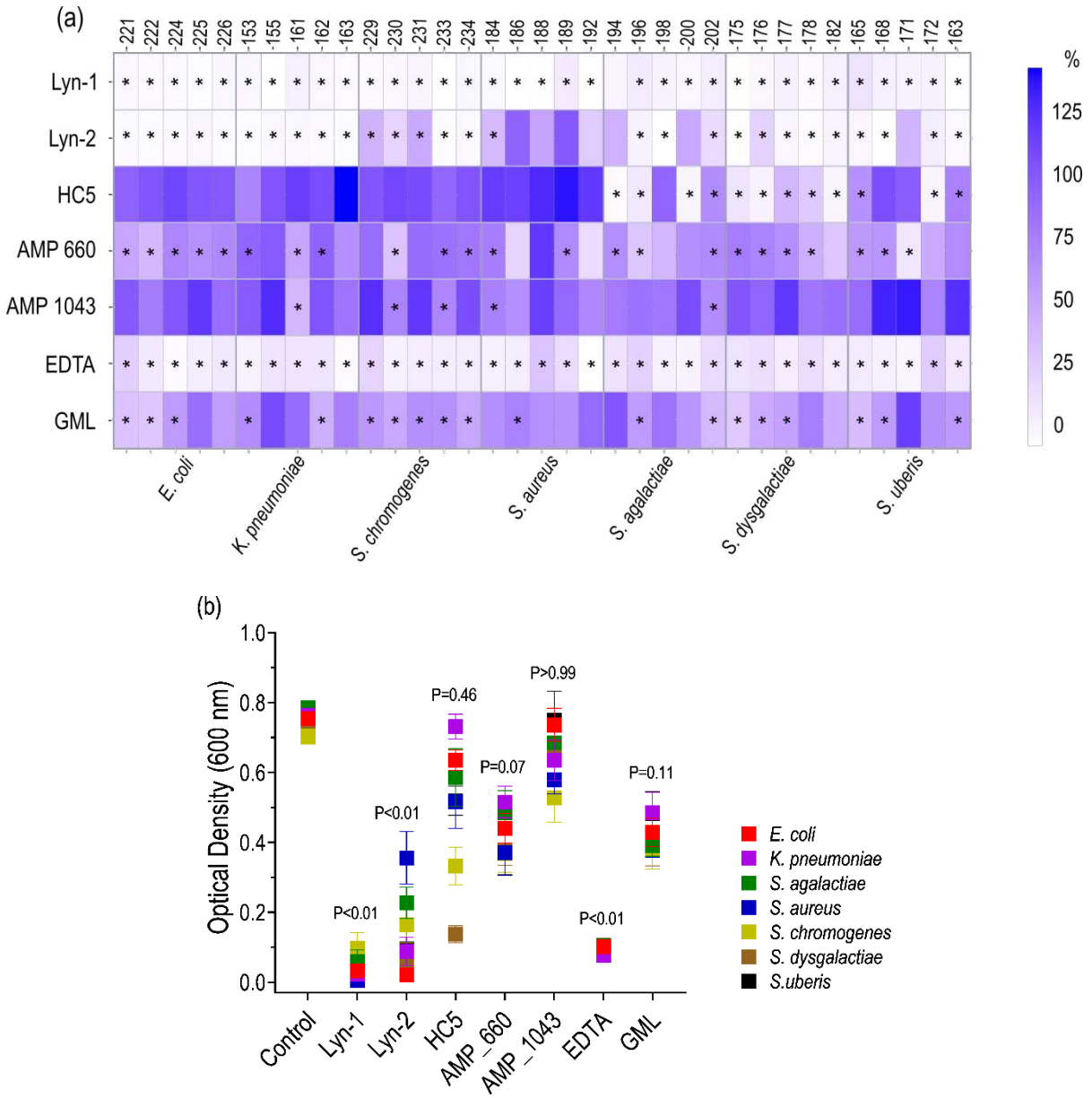

Figure 1: Antimicrobial activity of AMPs, EDTA, and GML against bovine mastitis pathogens. a) Heat map showing the inhibition (%) of mastitis pathogens by AMPs (128 mg/L), EDTA (2.7 g/L), and GML (2.2 g/L). Numbers across the top of the heat map represent strain ID. The color shade of each cell corresponds to the strain-specific growth percentual in the presence of antimicrobials, according to the sidebar legend. Asterisks represent a significant ($P < 0.05$) inhibition of bacterial growth compared to the control (Multiple Mann-Whitney test). b) Boxplot showing differences in the activity of AMPs (128 mg/L), EDTA (2.7 g/L), and GML (2.2 g/L) against mastitis pathogens. Each box corresponds to the average maximum optical density at 600 nm of five different strains, grouped by species, as indicated in the color-coded legend. Bars show the standard error of the mean.

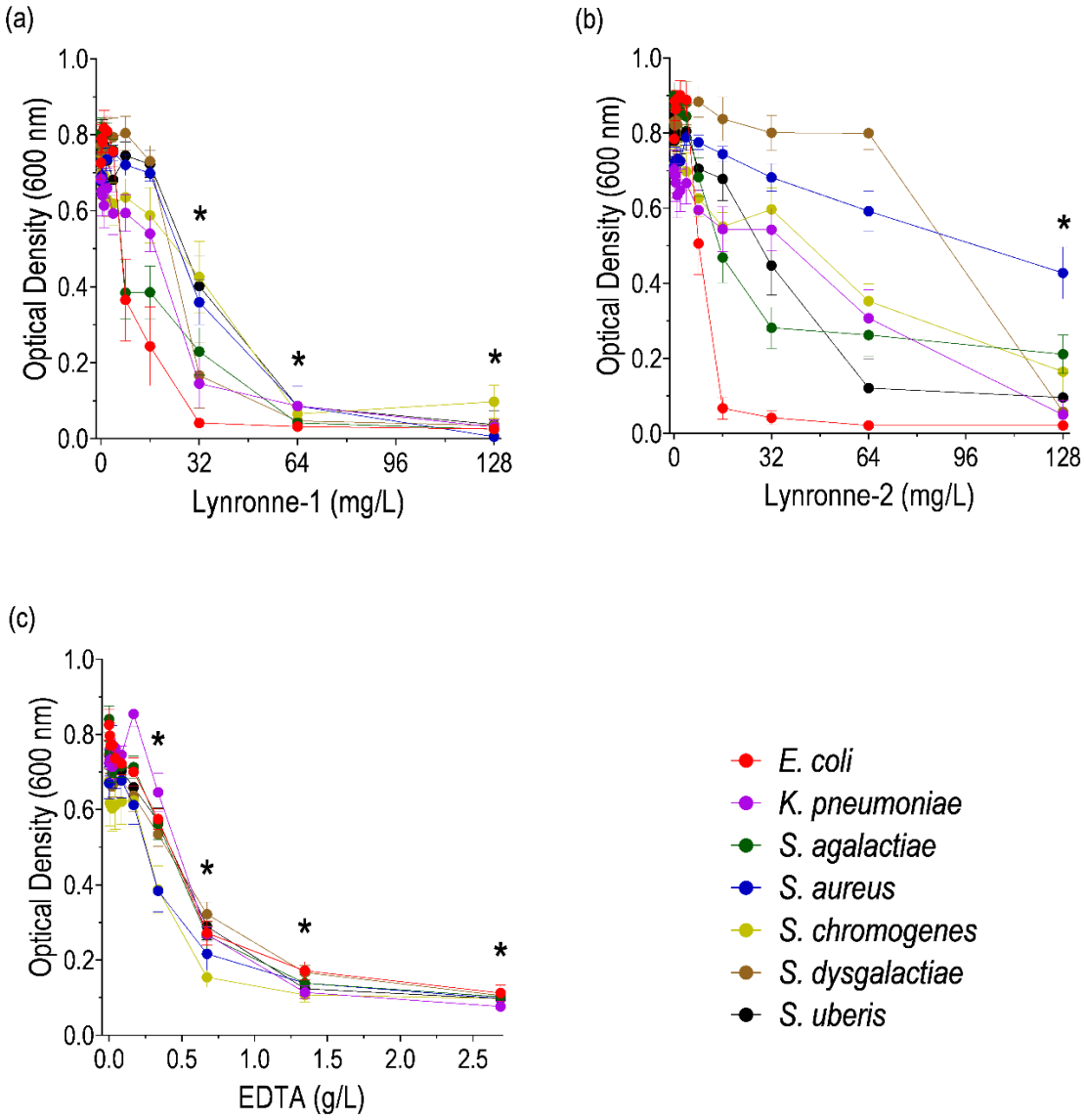

Figure 2: Concentration-dependent effect of lyn-1 (a), lyn-2 (b), and EDTA (c) on the growth (OD_{600}) of bovine mastitis pathogens. Dots represent the mean OD_{600} of the five strains for each bacteria group. Each species is color-coded as indicated in the figure legend. Bars show the standard error of the mean. Asterisks indicate a significant difference ($P < 0.05$) for designated concentrations compared to the control.

Antimicrobial synergy assays

The checkerboard approach was employed to evaluate the combined effects of lyn-1 and EDTA. To assess the interactive effect of the antimicrobial combination, the Fractional Inhibitory Concentration Index (FICI) was calculated. FICI results demonstrated a synergistic interaction between lyn-1 and EDTA, with values ranging from 0.1 to 0.5 for specific strains

across different bacterial species (Fig. 3). Strain-to-strain variation was observed within each bacterial group. Overall, the combination was highly synergistic against streptococci and *E. coli* (FIC range 0.1-0.3), but also showed a strong interactive synergistic effect (FIC range 0.2-0.5) against *S. aureus*, coagulase-negative Staphylococci and *K. pneumoniae* (Fig. 3). For example, these synergies decreased by 6-fold and 32-fold the concentration of lyn-1 required to inhibit the growth of *S. aureus* and *S. agalactiae*, respectively. In addition, there were no significant differences between FIC values across bacterial groups (ANOVA, $P > 0.05$).

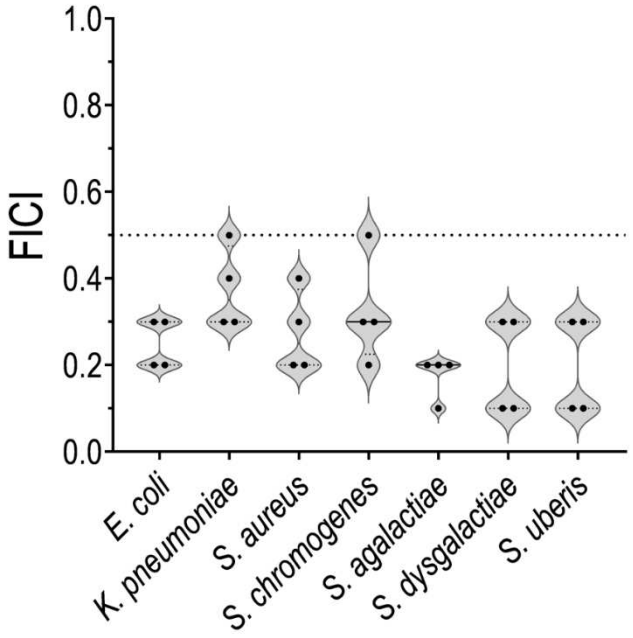

Figure 3: Synergistic effect of the lyn-1-EDTA combination on different species of mastitis pathogens. The dotted line indicates the synergism threshold with FICI ≤ 0.5.

The synergism between lyn-1 and EDTA was refined beyond the endpoint values (MIC) of each compound in the combination by using isobologram analysis, which allows a graphical representation of the relative potency of two-drug combinations (Fig. 4). The inflection point on the isobole equation curve indicates optimal binary mixtures, corresponding to the combination antimicrobials with the smallest FICI value. For *K. pneumoniae* and *S. aureus*, the most synergistic combination was obtained with 32 mg/L of lyn-1 and 1.02 g/L of EDTA. For *E. coli*, the optimal antimicrobial interaction consisted of 8 mg/L of lyn-1 and 2.04 g/L of EDTA, while *S. dysgalactiae* was effectively inhibited by a combination of 8 mg/L of lyn-1 and 1.02 g/L of EDTA. For *S. agalactiae*, a high synergistic effect was achieved with 4 mg/L of lyn-1 and 0.51 g/L of EDTA (Fig. 4). For applied purposes, these synergies indicate

that the concentrations of the two interacting molecules can be reduced by one quarter (lyn-1) or one eighth (EDTA) to achieve effective antibacterial activity. Importantly, these effects were not strain-dependent, and even cultures containing a mixture of different bacterial strains were consistently inhibited by lyn-1 and EDTA combinations (Fig. 4).

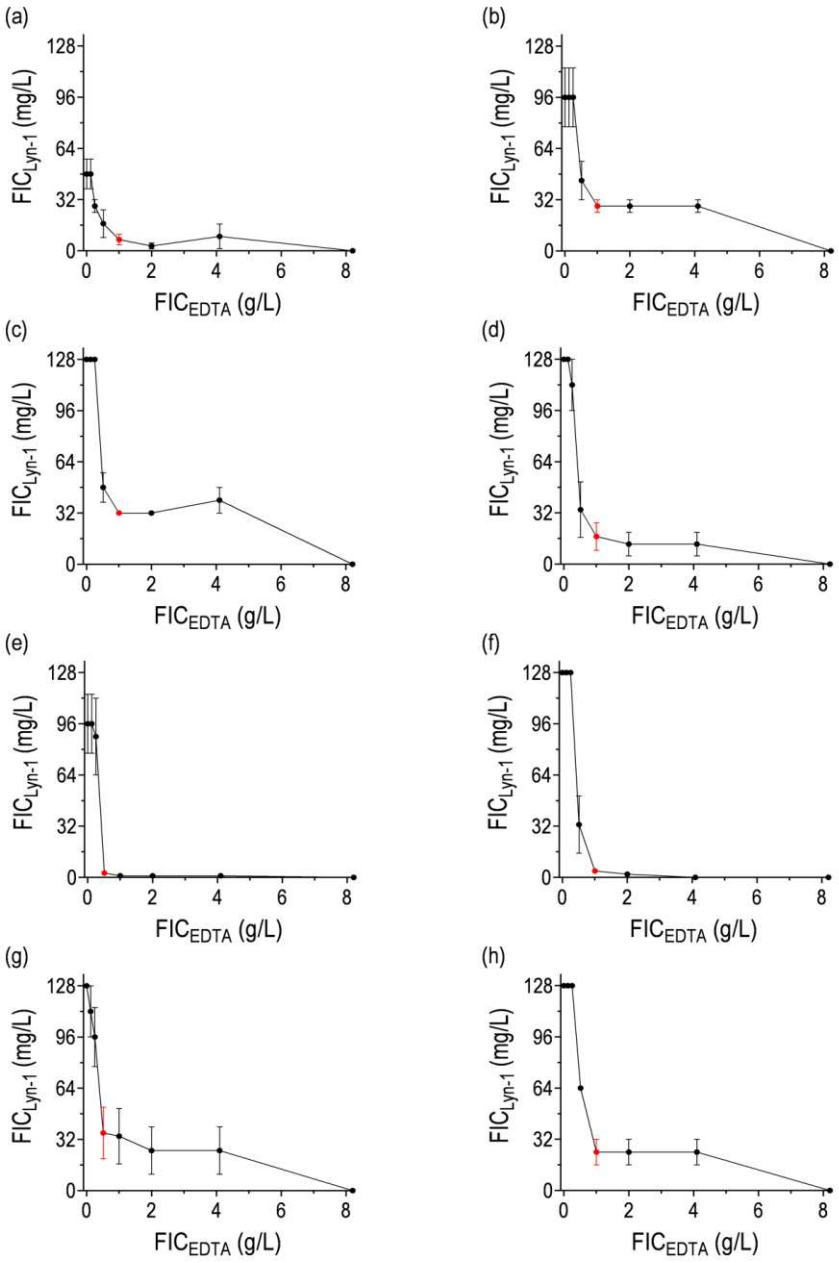

Figure 4: Isobolograms for combinations of lyn-1 and EDTA. Each panel represents a pool of strains (n = 5) grouped by species. Red points indicate the point of inflection on each graph. Bars show the standard error of the mean. a) *Escherichia coli* strains. b) *Klebsiella pneumoniae* strains. c) *Staphylococcus aureus* strains. d) *Staphylococcus chromogenes* strains. e) *Streptococcus agalactiae*

strains. f) *Streptococcus dysgalactiae* strains. g) *Streptococcus uberis* strains. h) Pool of all strains in equivalent amounts (n = 35).

Time-dependent killing assays

The efficacy of antimicrobials as a function of time was tested against *K. pneumoniae* 161 and *S. aureus* 189, which were selected among other strains based on their higher MIC to lyn-1 and to represent the Gram-negative and Gram-positive mastitis pathogens, respectively. Lyn-1 alone at a 1x MIC concentration (128 mg/L) drastically decreased the viability of *K. pneumoniae* after 45 minutes to 6 hours of exposure, with limited bacterial regrowth observed at 12 hours (Fig. 5a). Overall, a 5.36 log-cycles reduction in the bacterial population was observed within 24 hours of exposure to lyn-1, which represented a 91.5% reduction ($P < 0.05$) of the time-kill area under the curve (AUC) compared to the control (Fig. 5b). EDTA alone (8.2 g/L) only caused a 0.78 log-cycles decrease in the *K. pneumoniae* population relative to the control after 24 hours of incubation, indicating that its activity was mainly bacteriostatic (Fig. 5a). Consequently, the AUC of the EDTA treatment only reduced 13.2% ($P > 0.05$) and was not significantly different from the control (Fig. 5b).

The combination of lyn-1 and EDTA at a 1x MIC concentration (32 mg/L and 1.02 g/L of lyn-1 and EDTA, respectively) led to a 4.24 log-cycles reduction in the *K. pneumoniae* population after 24 hours of incubation. In contrast, the combination at 2x MIC concentration (64 mg/L and 2.04 g/L of lyn-1 and EDTA, respectively) caused a 6.30 log-cycles decrease in bacterial population relative to the control after 24 hours of incubation (Fig. 5a). The AUCs of both combinations represented a reduction ($P < 0.05$) of 76.6% and 99.6% for the 1x and 2x treatments, respectively. Statistical analyses also revealed that the treatments were not only significantly different from the control but also equivalent to lyn-1 treatment alone at a lower concentration ($P > 0.05$) (Fig. 5b).

When *S. aureus* 189 was exposed to lyn-1 alone at 128 mg/L, an initial decrease in bacterial viability occurred within 6 hours of treatment, but regrowth was evident after 12 hours and increased to 7.86 x 104 CFU/mL after 24 hours of incubation, representing a 3.33 log-cycles reduction (Fig. 5c). Nonetheless, the lyn-1 treatment represented a 60.3% decrease ($P < 0.05$) in the time-kill area under the curve (AUC) compared to the untreated cells (Fig. 5d). *S. aureus* cultures were killed by EDTA alone at 8.2 g/L, and a 3.41 log-cycles decrease in bacterial population was observed after 24 hours of incubation (Fig. 5c), which corresponded

to a reduction of 76.0% ($P < 0.05$) in the time-kill area under the curve (AUC) compared to the control (Fig. 5d).

The combined application of lyn-1 and EDTA at a 1x MIC concentration (32 mg/L and 1.02 g/L of lyn-1 and EDTA, respectively) decreased the *S. aureus* population by 3.76 log-cycles after 24 hours of incubation. Additionally, the combination at 2x concentration (64 mg/L and 2.04 g/L of lyn-1 and EDTA, respectively) induced a 4.03 log-cycles decrease in the bacterial population relative to the control after 24 hours of incubation (Fig. 5c). Both combinations exhibited significant killing efficacy ($P < 0.05$), with AUC reductions of 83.4% and 88.0% for 1x and 2x, respectively. Furthermore, the combination at 1x was equivalent to lyn-1 treatment alone ($P > 0.05$), albeit using one-quarter of the peptide concentration (Fig. 5d).

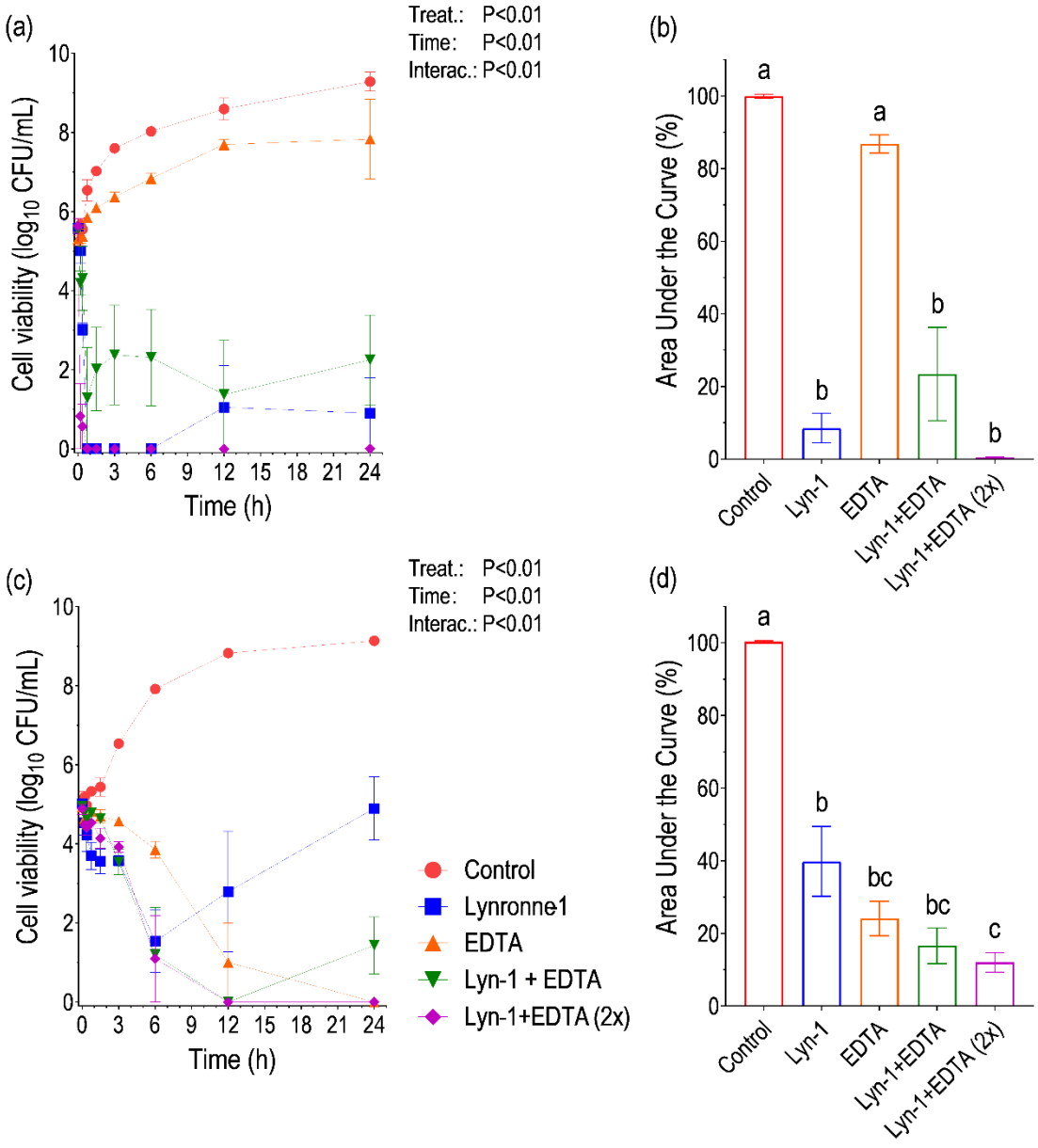

Figure 5: Bacterial viability and area under the curve (%) of *Klebsiella pneumoniae* (a, b) and *Staphylococcus aureus* (c, d) treated with lyn-1, EDTA, and their combinations. Cultures were grown in MH broth and treated with 128 mg/L of lyn-1, 8.19 g/L of EDTA, a combination of lyn-1-EDTA at 32 mg/L and 1.0 g/L, respectively, or a combination lyn-1-EDTA (2x) at 64 mg/L and 2.04 g/L respectively. Bars show the standard error of the mean. P-values denote Two-way ANOVA results. The AUC of the control was set to 100% and data were normalized by the growth control for comparison purposes. Different lowercase letters indicate statistical differences assessed by ANOVA followed by Tukey's test ($P < 0.05$).

Image processing and analysis

The dynamic changes in bacterial biomass (*K. pneumoniae* 161 and *S. aureus* 189) over time in response to lyn-1, EDTA, and 1x or 2x MIC combinations are represented in Fig. 6. The processed image analyses were conducted at 11 discrete time intervals (0 to 24 hours), with the initial culture at time 0 serving as the baseline reference at 100%. For *K. pneumoniae* 161, lyn-1 induced a rapid reduction in bacterial biomass, plummeting to 0.13% within 45 minutes (0,75 h) and complete inhibition (no colony formation) after 3 hours ($P < 0.05$). In contrast, EDTA-treated cultures maintained a high biomass production, peaking at 120% after 45 minutes, and maintained consistent growth throughout the 24-hour period. Lyn-1-EDTA 1x MIC treatment showed a gradual decline in biomass, with a significant decrease of 11% observed after 22 minutes (0.36 hours), while lyn-1-EDTA 2x MIC treatment maintained consistently low growth, dropping to 2.2% after 11 minutes (0.18 hours) (Fig. 6a and 6b).

In the case of *S. aureus* (Fig. 6c and 6d), lyn-1 treatment induced a consistent reduction in bacterial biomass, reaching 60.9% after 1.5 hours ($P < 0.05$), 0.65% at 6 hours and recovering to 20.4% by 24 hours. EDTA treatment exhibited initial fluctuation and subsequently led to a steady reduction, reaching complete growth eradication at 12 hours (Fig. 6c). Lyn-1-EDTA 1x MIC and lyn-1-EDTA 2x MIC treatments followed similar patterns, initially peaking at 22 minutes (128.9% and 116.4%, respectively), followed by a continuous decline in bacterial biomass after 1.5 hours to zero after 12 hours, except for a marginal increase in the 1x MIC combination after 24 hours (Fig. 6c and d).

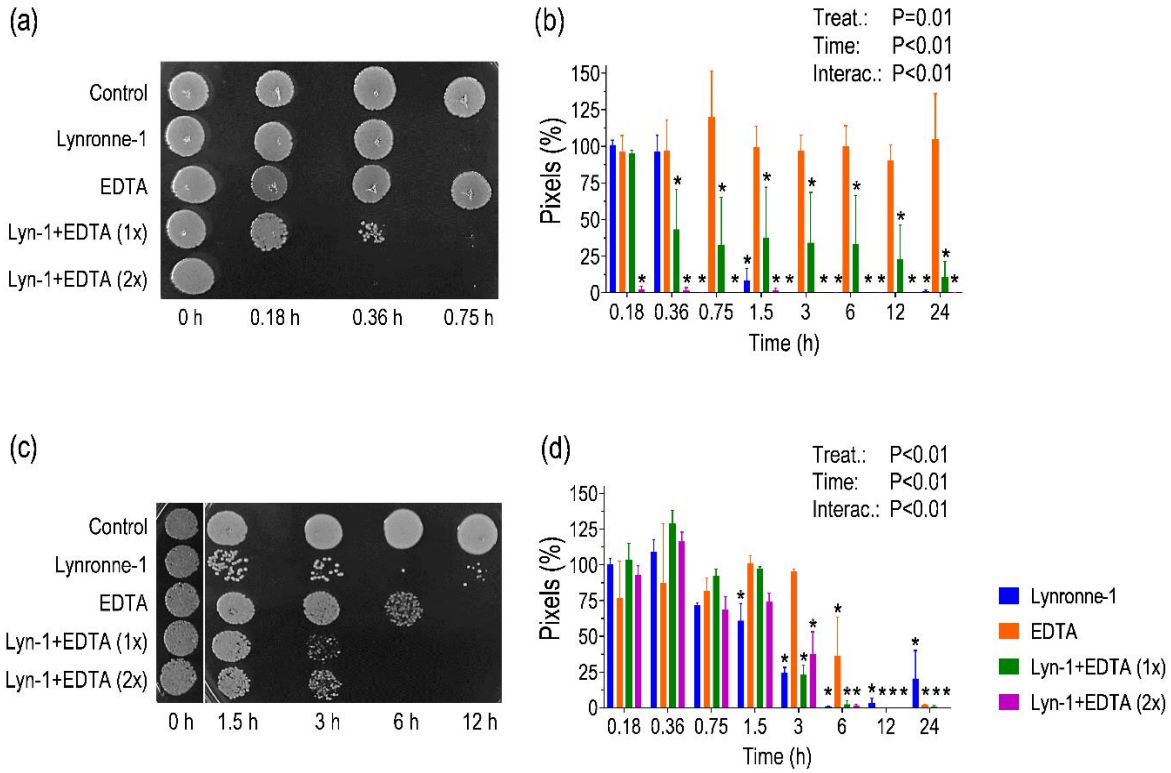

Figure 6: Effect of different antimicrobial treatments on biomass production by *K. pneumoniae* 161 and *S. aureus* 189. a) Biomass production by *Klebsiella pneumoniae* 161. b) Pixel analysis of *K. pneumoniae* growth. c) Biomass production by *Staphylococcus aureus* 189. d) Pixel analysis of *S. aureus* 189 growth. Error bars (b and d) represent the standard error of the mean. P-values denote Two-way ANOVA results. Asterisks show a significant difference ($P < 0.05$) in bacterial growth between treatment and control, assessed by Dunnet's test.

Determination of hemolytic activity

EDTA and the dose-reduced combination of lyn-1-EDTA (32 mg/L and 1.0 g/L, respectively) were non-hemolytic against bovine erythrocytes. When concentrations ranged from 1x to 8x MIC, neither EDTA nor the combination exceeded the auto-hemolysis ratio threshold of 14%. Lyn-1 alone also displayed low toxicity against bovine erythrocytes and some permeabilization of eukaryotic membranes (<29%) was only observed ($P > 0.05$) at supra-MIC concentration (1 g/L) (Fig. 7).

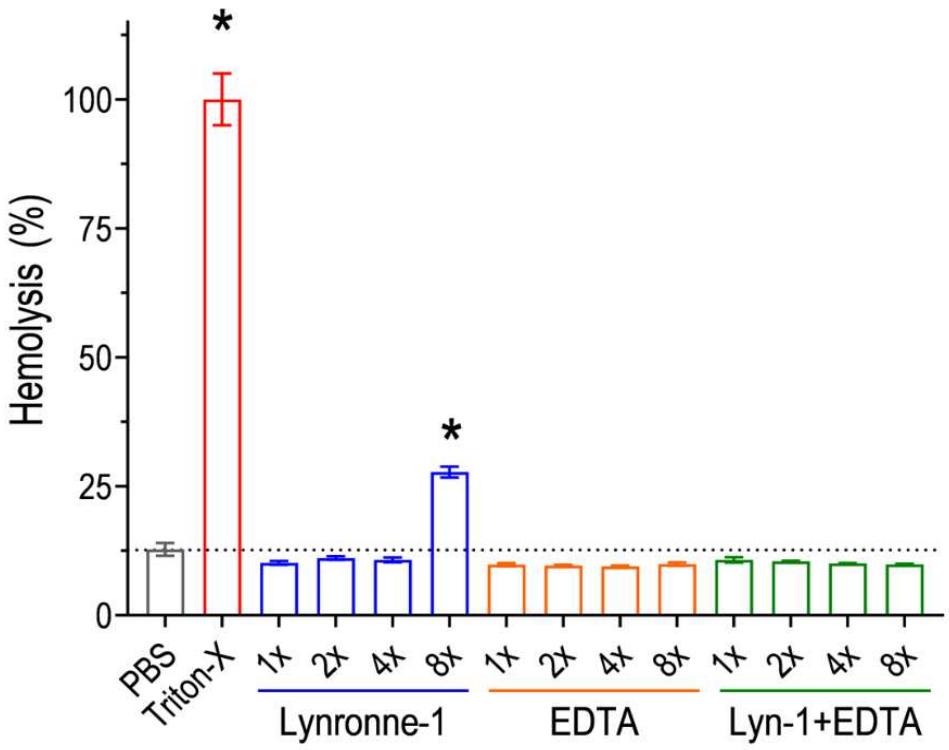

Figure 7: Hemolytic effect of lyn-1, EDTA, and their combination. Washed bovine erythrocytes were exposed to different concentrations of the antimicrobials for 1 hour. Individual antimicrobials were tested at multiple MIC: 1x, 128 mg/L for lyn-1 and 8.19 g/L for EDTA; 2x, 256 mg/L for lyn-1 and 16.4 g/L for EDTA; 4x, 512 mg/L for lyn-1 and 32.7 g/L for EDTA; and 8x, 1.02 g/L for lyn-1 and 65.5 g/L for EDTA. For the antimicrobials in combination, the dose-reduced MIC was defined as follows: 1x, 32 mg/L for lyn-1 and 1.02 g/L for EDTA; 2x, 64 mg/L for lyn-1 and 2.04 g/L for EDTA; 4x, 128 mg/L for lyn-1 and 4.09 g/L for EDTA; and 8x, 256 mg/L for lyn-1 and 8.19 g/L for EDTA. Asterisks show a significant difference ($P < 0.05$) in hemolysis between treatment and control (PBS), assessed by the Kruskal-Wallis test followed by Dunn's test. The dotted line indicates the occurrence of natural hemolysis indicated by PBS control.

Cytotoxicity of the lyn-1-EDTA combination against epithelial bovine mammary alveolar cells

Lyn-1 exhibited toxicity against the MAC-T cells, but only when concentrations exceeded 2x FIC. Cell viability was above 100% at 1x and 2x FIC and dropped to ~ 20% at 4x and 8x FIC. Similarly, MAC-T cells treated with disodium EDTA displayed viability >130%

at 1x FIC, 54.2% at 2x FIC, and was lower than 30% at 4x and 8x FIC. The combination of lyn-1 and EDTA showed little toxicity at 1x MIC concentration, with cell viability >92%. However, the viability of eukaryotic cells decreased to <20% at concentrations 2x MIC and above (Fig. 8a). Observations of MAC-T cells post-treatment revealed distinctive cellular responses. Lyn-1 treatment reduced cell viability at concentrations above the FIC while preserving cell size, shape, and confluence (Fig. 8b). EDTA exposure led to marked alterations in cell morphology to a rounded, shrunken appearance and with complete loss of confluence, even at lower concentrations. Additionally, cell count visibly decreased with increasing EDTA concentration. Combining lyn-1 and EDTA resulted in similar morphological changes, including disrupted confluence, cell shrinkage, and membrane blebbing by 1x MIC, leading to eventual cell lysis at the highest (8x MIC) concentration (Fig. 8b).

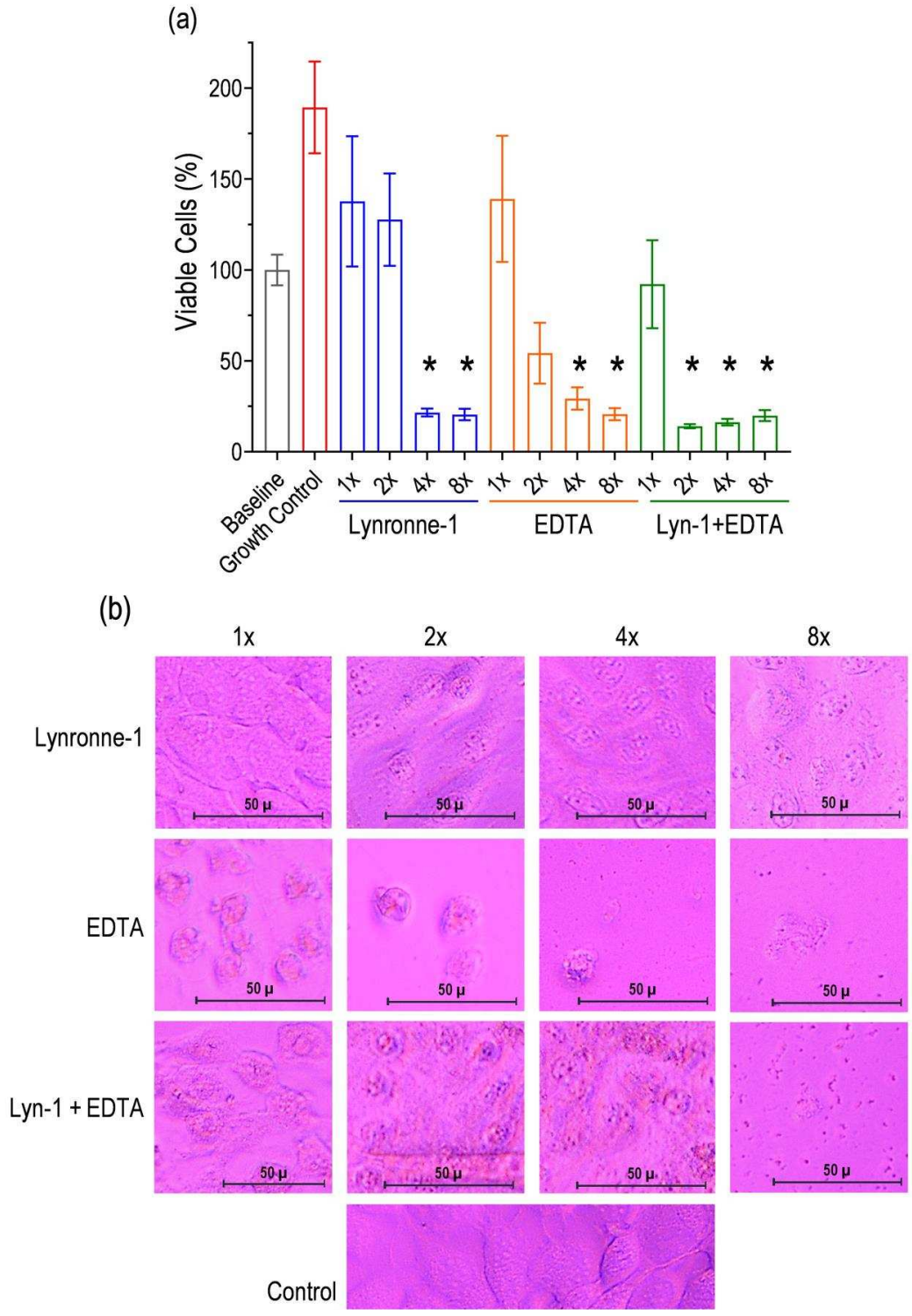

Figure 8: Effect of lyn-1, EDTA, and the lyn-1-EDTA combination on the viability of epithelial bovine mammary alveolar (MAC-T) cells. Individual antimicrobials were tested at the Fractional Inhibitory Concentration (FIC) values: 1x, 32 mg/L for lyn-1 and 1.02 g/L for EDTA; 2x, 64 mg/L for lyn-1 and

2.04 g/L for EDTA; 4x, 128 mg/L for lyn-1 and 4.09 g/L for EDTA; 8x, 256 mg/L for lyn-1 and 8.19 g/L for EDTA. The lyn-1-EDTA treatment represents the FICs combined at the respective values. a) Barplot showing the effect of the antimicrobials at different concentrations against MAC-T cells after 16 hours of treatment. Asterisks represent a significant difference ($P < 0.05$) in cell viability rate between treatment and control (PBS), assessed by the Kruskal-Wallis test followed by Dunn's test. b) Photomicrographs from light microscopy of MAC-T cells after 16 h of exposure to different concentrations of FICs each antimicrobial alone or their combination at the dose-reduced concentrations.

DISCUSSION

In this work, we investigated, for the first time, the synergistic combination of a rumen microbiome-derived AMP and an FDA-approved chelating agent as a non-antibiotic alternative for controlling bovine mastitis, the costliest infectious disease in the dairy industry. Initially, we screened the antimicrobial efficacy of five AMPs (lynronne-1, lynronne-2, bovicin HC5, AMP 1043, and AMP 660), and two known bioactive compounds (disodium EDTA and Glycerol monolaurate) against a panel of 35 strains of bovine mastitis pathogens. Results demonstrated that lyn-1 and EDTA have broad-spectrum activity against different species of bacteria frequently associated with contagious and environmental mastitis. Lyn-2 also exhibited high inhibitory activity against most tested species, except for *S. aureus*. Additionally, AMP 660 and GML showed promising activity against multiple strains, whereas bovicin HC5 exhibited activity mostly against *Streptococcus* strains. Only AMP 1043 had a limited effect against the mastitis pathogens. The combination of lyn-1 and EDTA caused at least a four-fold reduction in the MIC of the AMP (128 to 32 mg/L) for all strains tested (n = 35), and robust synergistic interactions were observed against multiple strains and species of mastitis bacteria, with an average FICI below the threshold of 0.5. The lyn-1-EDTA combination effectively prevented the regrowth of *S. aureus* in culture media and showed drastic killing activity (within minutes) against *K. pneumoniae*. The combination demonstrated non-hemolytic effects against epithelial bovine mammary gland cells up to eightfold its MIC (256 mg/L and 8.19 g/L for lyn-1 and EDTA, respectively). Unexpectedly, the safety evaluation of these compounds combined revealed cytotoxic effects on MAC-T cells at concentrations above 32 mg/L for lyn-1 and 1.02 g/L for EDTA.

Lynronne-1 is a linear α-helical AMP that was discovered through agar-based functional screening of a rumen bacterial metagenomic library and *in silico* analysis to predict AMP sequences (Oyama *et al.*, 2017). Lyn-1 activity is mediated by pore-formation in the cytoplasmic membranes of target cells and previous work using model membranes indicated a clear preference for interaction with lipids with an anionic headgroup, such as those found in the cytoplasmic membrane of bacteria (Jayawant *et al.*, 2021). In addition to their efficacy *in vitro*, lyn-1 has been shown to reduce bacterial counts in MRSA wound infections in a murine model. Importantly, prolonged exposure to sub-lethal doses of lyn-1 did not lead to decreased MRSA susceptibility, and no mutants resistant to the peptide could be isolated (Oyama *et al.*, 2017). This finding aligns with the results from the current study and underscores the broad-spectrum activity of lyn-1, as evidenced by its strong bactericidal activity against different species/strains of mastitis pathogens.

Broad antimicrobial activity was also observed for EDTA, a chelating agent with a high affinity for metal ions that has bacteriostatic properties (Finnegan and Percival, 2015; Hamoud, Reichling and Wink, 2015). It has been hypothesized that disodium EDTA may displace divalent cations in the bacterial cell envelope, specifically, Mg^{2+} and Mn^{2+}, which are essential for peptidoglycan biosynthesis and outer membrane stabilization (Farca, Nebbia and Re, 1993; Finnegan and Percival, 2015; Khan *et al.*, 2015). Therefore, EDTA can affect the integrity of the cell envelope in both Gram-negative and Gram-positive bacteria, thus facilitating the diffusion of hydrophobic molecules into the cytoplasmic membrane, including antimicrobials with amphipathic properties like AMPs (Khan *et al.*, 2015). As such, EDTA has been used in combination with some traditional antibiotics to overcome bacterial resistance by promoting changes in the permeability of the bacterial envelope of target cells (Lambert, Hanlon and Denyer, 2004; Hamoud, Reichling and Wink, 2015; Shahiwala, Khan and Bostanooei, 2017). Additionally, the capacity of EDTA to sequester essential metal ions like calcium, magnesium, zinc, and iron makes it a promising agent for biofilm control. (Finnegan and Percival, 2015).

Due to these features, a few studies have investigated the potential synergistic effects of EDTA in combination with various antibacterial agents. For example, Schlievert and Peterson (2012) reported that EDTA combined with GML demonstrated enhanced antimicrobial activity against resistant strains of *Pseudomonas aeruginosa* and Enterobacteriaceae. A study exploring the combination of EDTA, sanguinarine, and streptomycin, reported synergistic inhibitory activity against various bacteria, including

multidrug-resistant strains (Hamoud, Reichling and Wink, 2015). Furthermore, a combination of EDTA, levofloxacin, and chitosan was effective in reducing antibiotic-resistant MRSA infections both *in vitro* and *in vivo*, showcasing the potential of these combinations in overcoming antibiotic resistance (Shahiwala, Khan and Bostanooei, 2017). EDTA also exhibits synergistic effects when combined with AMPs, particularly towards Gram-negative bacteria, due to their impermeable outer membrane. Disodium EDTA (6.7-10.0 g/L) enhanced the spectrum of activity of nisin, a natural antimicrobial peptide with GRAS status used as a food preservative, against both *E. coli* and *Salmonella Typhimurium* (Khan *et al.*, 2015) Additionally, a separate study reported that bovicin HC5, a lantibiotic produced by a ruminal *Streptococcus*, showed bactericidal activity against *Salmonella Typhimurium*, but only when combined with EDTA (0.46 g/L) (Prudencio, Mantovani and Vanetti, 2014; Prudêncio *et al.*, 2016). This current study is the first to use checkerboard and isobologram analysis to show synergistic interactions between lyn-1 and EDTA, with FICI values ranging from 0.1 to 0.5 for different bacterial species. The potent activity of the combination against major bacterial species associated with clinical and environmental bovine mastitis is promising for the development of AMP-based therapeutic applications.

Nonetheless, the assessment of toxicity is also a crucial step in evaluating the potential clinical application of antimicrobial therapies. Previously, lyn-1 was shown to have minimal hemolytic activity against human red blood cells and negligible cytotoxicity both *in vitro* and *in vivo* (Oyama *et al.*, 2017; Greco *et al.*, 2020; Adam J Mulkern *et al.*, 2022). As pointed out above, results from the current study confirm that lyn-1 also has little hemolytic activity against bovine erythrocytes, which agrees with these previous observations. Moreover, when combined with EDTA, a well-known anticoagulant, lyn-1 did not cause significant hemolysis of bovine red blood cells even at the highest concentration tested in the combination (256 mg/L), which reinforced its safety attributes and limited capacity to cause hemolysis.

The cytotoxic effects observed against the MAC-T cells were unexpected and contrasted with the observed low specificity for mammalian cells and greater selectivity for bacterial cells reported for lyn-1 (Oyama *et al.*, 2017; Jayawant *et al.*, 2021). However, some variation in the survival rate of mammalian cells exposed to lyn-1 has been reported. Although lyn-1 showed low toxicity against human umbilical vein endothelial cells (HUVEC) and hepatocellular carcinoma cells (Hep G2) (Oyama *et al.*, 2017) the viability of human bronchial fibroblasts (IMR90) and human lung epithelial cells (BEAS-2B) were significantly reduced by lyn-1 at an IC50 of 94.23 and 138.9 mg/L, respectively. However, these toxic effects were not

observed when the peptide was administered to animals or an invertebrate model to control experimentally induced bacterial infections (Oyama *et al.*, 2017; Adam J Mulkern *et al.*, 2022). Using a murine model of MRSA skin infection, topical administration of lyn-1 at 2% or 10% (w/v) led to a significant reduction in bacterial load relative to the control group, confirming therapeutic efficacy without toxic effects. Additionally, no systemic toxic effects were reported for test animals that received intravenous administration of lyn-1 at 10 mg/kg subsequently to a deep thigh infection with an MRSA strain (Oyama *et al.*, 2017). Importantly, the intravenous treatment did not yield the expected antimicrobial effects, which was explained by the low stability of the peptide in the serum, indicating susceptibility to proteolytic degradation (Oyama *et al.*, 2017). Lyn-1 also did not show toxic effects in a *Galleria mellonella* infection model when the larvae were treated with the peptide at concentrations ranging from 32 mg/kg to 96 mg/kg. Notably, lyn-1 showed a protective effect by preventing melanization and death of larvae inoculated with *Pseudomonas aeruginosa* PAO1 (Mulkern *et al.*, 2022). These observations indicate that the toxicity assessment of antimicrobial peptides *in vitro* might reveal adverse effects that do not fully mimic the physiological conditions and tolerance of mammalian cells and tissues *in vivo*. Although the results presented in the current study cannot be ignored, a previous study reported discrepancies between *in vitro* toxicity data for antimicrobial peptides and the effects observed *in vivo*, which could lead to potentially inaccurate conclusions about the safety of promising antimicrobials (Greco *et al.*, 2020).

Similarly, the effects caused by EDTA on MAC-T cells might be related to the removal of calcium ions essential for maintaining integrin function and cell adhesion, as EDTA can act to promote cell detachment (Lai *et al.*, 2022). EDTA also contains four carboxylic groups and ionic dissociation leads to an accentuated decrease in pH, which can also impact cell viability. Consequently, the colorimetric MTT assay may pose a limitation in assessing cytotoxicity to eukaryotic cells, as EDTA could induce cell detachment during the wash steps, leading to reduced cell viability *in vitro*. Hence, like lyn-1, the cytotoxic effects observed for EDTA *in vitro* might be less evident at the tissue level due to the physiological conditions *in vivo*. Disodium EDTA has been utilized for over 50 years in chelation therapy to treat atherosclerosis. In a study by Lamas *et al.* (2013), patients received infusions at 6.0 g/L of EDTA weekly. Moreover, EDTA has been incorporated into marketed products for wound treatment at concentrations ranging from 0.5 to 10% w/w. Its cation-chelating feature is believed to inhibit enzymatic activity contributing to wound inflammation, and it also removes iron, which is crucial for microbial virulence and pathogenicity (Percival, Bowler and Parson, 2005).

CONCLUSION

Our current study shows that lyn-1 and the lyn-1-EDTA combination have strong bactericidal activity against a range of pathogens associated with bovine mastitis, a critical and costly disease for the dairy sector. Lyn-1 synergistically interacts with EDTA, and the combined antimicrobials have greater killing efficacy than each antimicrobial alone. Although toxic effects against epithelial bovine mammary alveolar (MAC-T) cells were observed at concentrations above MIC, evaluation of the susceptibility of bovine red blood cells to either lyn-1 or EDTA indicated little or no hemolytic activity. It is worth noting that previous studies have demonstrated safe effects in mouse models, demonstrating the potential discrepancy between *in vitro* and *in vivo* results. In summary, we show that lyn-1 and the lyn-1-EDTA combination have therapeutic potential in udder infections, providing alternative solutions to mastitis treatment.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1: Minimum Inhibitory Concentrations (MICs) against mastitis pathogens.

SPECIES	ID	Treatment (g/L)							
		CEF	Lyn-1	Lyn-2	HC5	660	AMP 1043	AMP EDTA	GML
Klebsiella pneumoniae	153	0.5	32	128	>128	>128	>128	2689	>2195
	155	0.25	64	128	>128	>128	>128	>2689	>2195
	161	0.25	128	128	>128	>128	>128	>2689	>2195
	162	0.25	32	128	>128	>128	>128	>2689	>2195
	163	0.25	32	64	>128	>128	>128	1344	>2195
Streptococcus uberis	165	0.5	32	64	>128	>128	>128	1344	>2195
	168	2	64	128	>128	>128	>128	1344	>2195
	171	4	64	>128	>128	128	>128	1344	>2195
	172	2	64	64	16	>128	>128	>2689	>2195
	173	4	128	64	>128	>128	>128	>2689	>2195
Streptococcus dysgalactiae	175	1	64	128	16	>128	>128	>2689	>2195
	176	2	32	>128	32	>128	>128	>2689	>2195
	177	1	32	128	4	>128	>128	2689	>2195
	178	1	32	128	4	>128	>128	>2689	>2195
	182	1	32	128	4	>128	>128	2689	>2195
Staphylococcus aureus	184	0.25	64	>128	>128	>128	>128	672.25	>2195
	186	0.25	64	>128	>128	>128	>128	2689	>2195
	188	0.25	64	>128	>128	>128	>128	>2689	>2195
	189	0.5	128	>128	>128	>128	>128	>2689	>2195
	192	0.5	64	>128	>128	>128	>128	336.12	>2195
Staphylococcus chromogenes	194	8	64	>128	32	>128	>128	672.25	>2195
	196	2	64	128	16	>128	>128	>2689	>2195
	198	2	64	128	>128	>128	>128	672.25	>2195
	200	32	128	>128	8	>128	>128	1344	>2195
	202	2	64	>128	>128	>128	>128	>2689	>2195
Escherichia coli	221	1	32	16	>128	>128	>128	>2689	>2195
	222	1	16	16	>128	>128	>128	2689	>2195
	225	1	32	16	>128	>128	>128	2689	>2195
	226	1	8	16	>128	>128	>128	>2689	>2195
	224	1	8	64	>128	>128	>128	672.25	>2195
Staphylococcus agalactiae	229	2	64	>128	>128	>128	>128	>2689	>2195
	230	2	32	>128	>128	>128	>128	2689	>2195
	231	4	64	>128	>128	>128	>128	2689	>2195
	233	1	32	32	>128	>128	>128	2689	>2195
	234	1	16	32	>128	>128	>128	2689	>2195

CHAPTER 2

ANTIMICROBIAL PEPTIDE LYNRONNE-1 AS AN ALTERNATIVE FOR COMBATING MASTITIS BIOFILMS

ABSTRACT

Mastitis, an inflammatory condition affecting the udders of dairy cattle, is one of the most common and costly diseases in the dairy industry worldwide. This study explores the efficacy of the antimicrobial peptide Lynronne-1 (Lyn-1) against established biofilms of major mastitis pathogens, including *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Staphylococcus chromogenes*. We assessed lyn-1's ability to prevent adhesion and invasion by these biofilm-forming pathogens using seven representative bacterial strains with high biofilm-forming capacities. The minimal inhibitory concentration (MIC) of Lyn-1 against biofilm-forming strains was determined to be 128 µg/mL. At this concentration, Lyn-1 reduced the average pre-formed biofilm by 50% in Gram-positive strains and 7.17% in Gram-negative strains. Notably, Lyn-1 significantly decreased the established biofilm of *S. aureus* 184 ($P < 0.05$) and consistently inhibited new biofilm formation across all strains. Adhesion and invasion assays using epithelial bovine mammary alveolar cells (MAC-T) showed a significant reduction ($P < 0.05$) in both adhesion and invasion rates at two different infection concentrations. Fluorescence microscopy indicated lyn-1's potential efficacy, with dead bacterial cells surrounding live eukaryotic cells. These results highlight lyn-1's potential as an effective and safe alternative for mastitis prevention and treatment.

Keywords: antimicrobial peptide, bovine mastitis, adhesion and invasion, biofilm

INTRODUCTION

Bovine mastitis is characterized by inflammation of the mammary gland and stands as the most prevalent disease affecting dairy cows worldwide (Cobirka, Tancin and Slama, 2020). Beyond its impact on animal health and well-being, mastitis presents a complex challenge for the dairy industry due to its association with chronicity and the escalating problem of antibiotic resistance (Kovačević *et al.*, 2022). Udder infections are primarily caused by bacterial pathogens, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli,* and emerging *Klebsiella pneumoniae*, and pose a significant threat to milk production, animal welfare, and economic viability of dairy farming (Silva *et al.*, 2021; de Campos *et al.*, 2023).

Conventional mastitis management often relies on antibiotics (Gomes and Henriques, 2016), with mastitis being the primary reason for antibiotic usage in the dairy sector (Stevens, Piepers and Vliegher, 2019). While antibiotics are effective in treating bovine mastitis, their efficacy varies among animals and bacterial strains. Factors that influence treatment success include lactation stage, treatment duration, pathology severity, and the immune response (McDougall *et al.*, 2019). However, the recurrent use of intramammary antibiotics may contribute to the selection of antibiotic-resistant strains. Additionally, antibiotic residues in milk can disrupt fermentation processes in the dairy industry or trigger allergic reactions in consumers (Kurjogi *et al.*, 2019).

The complexity of bovine mastitis is aggravated by the capacity of bacterial pathogens to form biofilms. Biofilms serve as protective environments, that shield bacteria from the host immune response and hinder the effectiveness of antibiotic treatments (Hathroubi *et al.*, 2017). In the context of dairy cattle, the formation of biofilms has been linked to chronic and recurring mastitis infections (Melchior, Vaarkamp and Fink-Gremmels, 2006), as it contributes to the evasion of immunological defenses and facilitates adherence and colonization of host gland epithelium (Baselga *et al.*, 1993; Oliveira *et al.*, 2006; Schönborn and Krömker, 2016). Moreover, the stress tolerance and slowed growth rate of sessile cells, coupled with the genetic heterogeneity prevalent within the biofilm bacterial population, significantly reduce susceptibility to antimicrobials (Prenafeta, 2014; Wang *et al.*, 2023) Therefore, addressing these factors is crucial for developing more effective strategies against bovine mastitis and bacterial pathogenesis.

Antimicrobial peptides (AMPs) possess potent antibiofilm properties by targeting various stages of biofilm formation and maintenance through diverse mechanisms (Hancock, Alford and Haney, 2021). Antibiofilm peptide LL-37 can inhibit the initial attachment of bacteria, even at sub-inhibitory concentrations (Hell *et al.*, 2010), while synthetic peptide 1037 impedes *Pseudomonas aeruginosa* motility, which is crucial for its surface attachment during biofilm formation (de la Fuente-Núñez *et al.*, 2012). Additionally, AMP 1018 exerts its antibiofilm activity by targeting the stringent stress response - a mechanism activated by bacteria in response to stressors to promote biofilm formation for survival. AMP 1018 binds with the second messenger molecule guanosine tetraphosphate, inducing its degradation. This process effectively halts biofilm growth and promotes dispersion (de la Fuente-Núñez *et al.*, 2014). Moreover, certain peptides, such as piscidin 3, act directly on the biofilm matrix disrupting biofilm architecture by degrading extracellular DNA, a critical matrix component, through binding with cationic copper (Libardo *et al.*, 2017).

A previous study reported that the antimicrobial peptide lynronne-1 (Lyn-1) was effective in controlling the proliferation of several bacterial pathogens (Oyama *et al.*, 2017). This peptide can form pores in the cell membrane of target bacteria and exhibit potent activity against a range of mastitis pathogens, including *E. coli, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcus chromogenes, Streptococcus agalactiae, Streptococcus dysgalactiae,* and *Streptococcus uberis*. Moreover, its antibiofilm activity has been demonstrated against methicillin-resistant *S. aureus* ATCC 33591 (Oyama *et al.*, 2017). Here we hypothesized that lyn-1 can inhibit biofilm formation by mastitis pathogens and prevent the bacteria from adhering and invading epithelial alveolar cells of the bovine mammary gland. Our results reveal that lyn-1 could help enhance the defense mechanisms of the mammary gland to protect against bacterial pathogenesis during infection.

MATERIAL AND METHODS

Bacterial Strains and Culture Conditions

In this study, a panel of 44 strains (25 Gram-positive and 19 Gram-negative bacteria), consisting of various species of major mastitis pathogens, was employed as target organisms. The panel included contagious and environmental staphylococci and streptococci, as well as *E. coli* and *Klebsiella pneumoniae*. These strains were derived from milk samples collected from

cows exhibiting clinical mastitis symptoms and submitted to the Wisconsin Veterinary Diagnostic Laboratory (WVDL) for routine diagnostics by accredited veterinarians. Strains were identified using Matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS; Bruker Daltonik; Bremen, Germany) and biochemical methods as outlined in the NMC Handbook (National Mastitis Council, 2017). Identification criteria included a score range of 2.3 to 3.0.

Biofilm quantification and target selection

The biofilm-forming capacity of the 44 strains was assessed using the crystal violet assay, following the methodology described by Stepanović *et al.* , (2000). Briefly, bacteria were cultivated at 37 ℃ for 48 hours in 96-well plates containing 200 µL of Brain Heart Infusion (BHI) broth enriched with 0.4% glucose. Subsequently, the medium was aspirated, and the wells were washed 3x with sterile Phosphate-buffered saline (PBS buffer). During the final wash, the plates were agitated to dislodge non-adherent bacteria. The remaining adherent bacteria were fixed using 200 µL of 99% methanol for 15 minutes. Next, the plates were emptied and allowed to air-dry in a biosafety cabinet. To stain the adherent cells, 200 µL of 2% crystal violet was applied for 5 minutes. The excess dye was then washed with distilled water. Once the plate was dry, the cells were resuspended with 200 µL of 33% glacial acetic acid, and the optical density at 570 nm (OD_{570}) was measured using a SpectraMax Plus 384® spectrophotometer (Molecular Devices, LLC., San Jose, USA). *Staphylococcus aureus* O46, a strain isolated from sheep with mastitis and recognized as a strong biofilm producer (Maréchal *et al.*, 2011), was used as a positive control.

Isolates were categorized as non-biofilm-former (NBF), weak biofilm-former (WBF), moderate biofilm-former (MBF) and strong biofilm-former (SBF), following the method proposed by Stepanovic *et al.* (2000). A cut-off value (ODcut) was defined as:

ODcut = ODblank + 3x standard deviation (SD) of ODblank;

Where:

i. $OD \leq ODcut$ = Non-biofilm-former (NBF)
ii. $ODcut < OD \leq 2 \times ODcut$ = Weak biofilm-former (WBF)
iii. $2 \times ODcut < OD \leq 4 \times ODcut$ = Moderate biofilm-former (MBF)
iv. $OD > 4 \times ODcut$ = Strong biofilm-former (SBF).

For subsequent assays, strains demonstrating the highest biofilm production were selected as targets.

Effect on bacterial growth

The effect of lyn-1 on bacterial growth was determined over a 24-hour period using a pool of biofilm-forming strains exposed to various concentrations of the peptide. Twofold serial dilutions of lyn-1 were added to individual wells within each plate row, with concentrations ranging from the previously determined Minimum Inhibitory Concentration (MIC) of 128 µg/mL down to 32 µg/mL. To prepare the inoculum of bacterial targets, the optical density of the cultures was individually adjusted to an OD_{600} of approximately 0.08 in cation-adjusted Mueller–Hinton broth (Millipore Sigma, Burlington, USA) using a Genesys30® spectrophotometer (Thermo Fischer Scientific, Madison, USA). Subsequently, the cultures were diluted 100-fold to a final concentration of 5×10^5 CFU/mL in MH broth for each well, and 20 µL of each strain was transferred to the 96-well plates. Incubation occurred at 37 °C for 24 hours, during which bacterial growth was monitored by measuring absorbance at 600 nm at 20-minute intervals using a microplate spectrophotometer SpectraMax Plus 384® (Molecular Devices, LLC., San Jose, USA).

Biofilm eradication activity

The capacity of lyn-1 to eradicate bacterial biofilms was assessed using the microdilution method in a sterile 96-well flat-bottomed plastic tissue culture plate. To establish the biofilm, the plates were inoculated with $1x10^5$ CFU/mL of a cell suspension prepared in BHI and enriched with 0.4% glucose. After incubation at 37 °C for 48 hours, the liquid in the wells was removed, and the biofilms were gently washed with PBS to eliminate non-adherent cells. The established biofilms were then exposed to lyn-1 (128 µg/mL, MIC) for 24 hours. The remaining biofilms were fixed using 99% methanol. Indirect quantification was carried out by staining with crystal violet. Plates were stained with 0.5% (w/v) crystal violet for 20 minutes, gently washed, and the bound crystal violet was solubilized by the addition of 33% (v/v) glacial acetic acid. Optical density was measured at 570 nm. The representation of biofilm formation was expressed as a ratio relative to the 48-hour biofilm control (Shukla and Rao, 2013; Wang *et al.*, 2015).

Epithelial bovine mammary alveolar cells and culture conditions

The Bovine Mammary Alveolar Cells (MAC-T) line, as documented by Huynh *et al.* in 1991, was selected for experimental investigations. The MAC-T cells were cultured in T75 cell culture flasks utilizing DMEM medium (Sigma-Aldrich, San Luis, USA), supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich, San Luis, USA), 100 U/mL penicillin, 10 mg/mL streptomycin (Sigma-Aldrich, San Luis, USA), and 5 g/mL insulin (Sigma-Aldrich, San Luis, USA). The cells were incubated at 37°C in a humidified incubator with 5% CO_2 and cultivated until a confluent monolayer was formed. The cells were then subjected to treatment with 0.05% trypsin (Sigma-Aldrich, San Luis, USA) as described by Sipos and Merkel (1970). Subsequently, the cells were resuspended in fresh MAC-T medium at a concentration of 10^5 cells/mL. For cytotoxicity assays, the cells were seeded into 96-well treated plates at a density of 10^5 cells per well and incubated overnight at 37°C in 5% CO_2 to achieve a confluent monolayer.

Prevention of bacterial adhesion and invasion

In this study, the effectiveness of Lyn-1 in preventing bacterial infections on MAC-T cells was assessed using pure cultures of seven different bacterial strains selected as biofilm formers. Each strain was cultured and assessed individually to determine its adhesion and invasion rates in the presence of Lyn-1. The assessment of antimicrobial efficacy in combination with an adherence and invasion assay was performed as described by Hensen *et al.* (2000). Bacterial strains were selected based on their biofilm-forming capacity, which included two *Escherichia coli*, two *Klebsiella pneumoniae,* two *Staphylococcus aureus*, and one *Staphylococcus chromogenes* strain. Cultures were routinely grown overnight in BHI media. Confluent monolayers of primary mammary epithelial cells, containing approximately 10^5 cells per well, were established in 96-well plates and washed at least three times with PBS. The final washing fluid was carefully removed, and 200 µL of DMEM media with lyn-1 at MIC concentration (128 µL/mL) were added. After 15 minutes, the bacterial inoculum was added to each well at a final concentration of approximately 10^5 CFU/mL and 10^7 CFU/mL, resulting in Multiplicities of Infection (MOIs) of 1:1 and 1:100, respectively. Plates were then incubated in an atmosphere of 5% CO_2 at 37°C. After 180 minutes of incubation, nonadherent bacteria were removed thoroughly by washing the plate at least 3x with PBS. To quantify adhered bacteria, cells were detached using sterile trypsin+EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA), and the number of attached bacteria was determined through viable counting using the drop plate technique for bacterial enumeration (Herigstad, Hamilton and

Heersink, 2001). For invasion studies, nonadherent bacteria were removed after 3 hours of incubation as described earlier. Following the removal of the last washing fluid, 100 µg/mL of gentamicin was added to kill extracellular bacteria. Plates were then incubated for 60 minutes at 37°C under 5% CO_2. The supernatant was discarded, and monolayers were washed thoroughly with PBS. Cell lysis was performed using Triton X-100 (0.1% vol/vol), and the number of bacteria invading the mammary gland epithelial cells was determined through enumeration of viable bacteria using the drop plate technique. Strains were assessed individually. Controls included wells without the addition of lyn-1 and wells without the addition of inoculum.

Viability of Bacterial Populations

To assess cell and bacteria viability post-treatment with lyn-1, fluorescence micrographs were captured following staining with the LIVE/DEAD™ BacLight™ Bacterial Viability Kit for microscopy and quantitative assays (Thermo Fischer Scientific, Waltham, USA). In this assay, live cells with intact membranes exhibit green fluorescence, while dead cells with compromised membranes fluoresce red. Adhesion assays were conducted as previously described, and after removing nonadherent bacteria, 3 µL of the dye mixture, consisting of equal volumes of SYTO 9 and propidium iodide, was added to co-cultures of bacteria and eukaryotic cells. *S. aureus* strain 184 was selected as the target due to lyn-1 demonstrating the most potent anti-biofilm activity against this strain.

The plates were incubated at room temperature in the dark for 15 minutes and subsequently visualized using a KEYENCE BZ-X800® microscope (Keyence Corporation, Itasca, USA). Excitation and emission were, respectively, 480/500 nm for the SYTO 9 (green) stain, and 490/635 nm for the propidium iodide (red) stain, as recommended by the manufacturer. Stained cells were observed at both 1000x and 400x magnification.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism 8.4.3 software (Motulsky, 2023). Data normality was assessed using the Shapiro-Wilk test. Differences were considered significant when $P < 0.05$ and were considered a trend when $P < 0.10$. Results represent the mean ± standard deviation.

For biofilm formation assays, datasets were normalized by subtracting the average OD_{570} of blanks. The Kruskal-Wallis test was then applied for analysis, followed by post-hoc Uncorrected Dunn's test for pairwise comparisons against the positive control. The experimental setup included two biological replicates and three technical replicates.

To evaluate the effect of antimicrobials on bacterial growth, the dataset was normalized by subtracting the average OD_{600} of blanks. Experimental assays were performed with three biological replications and two technical replicates. To assess differences between treatments, the Kruskal-Wallis test was applied for analysis of final OD_{600}, followed by Uncorrected Dunn's test for pairwise comparisons against the control. To assess differences in bacterial growth dynamics, the area under the curve (AUC) was calculated for each replicate. To evaluate the impact on established biofilms, datasets were normalized by subtracting the average OD_{570} of blanks, and the average OD_{570} of the baseline was adjusted to 100%. The experiment was conducted with four biological and three technical replicates. To assess the impact of lyn-1 on bacterial growth and established biofilms, ANOVA was performed on the AUC results and normalized biofilm data, followed by Tukey's test for pairwise comparisons to evaluate significant differences among the results. For prevention of bacterial adhesion and invasion assays, the experiment was performed twice and two technical replicates, and pairwise inferences were made using multiple t-tests.

RESULTS

Biofilm quantification and target selection

The biofilm-forming capacity of bacterial strains was evaluated after 48 hours of incubation in glucose-enriched media. The optical density at 570 nm (OD_{570}) for the tested strains ranged from 0 to 1.21. The positive control strain, *S. aureus* O46, which is considered a strong biofilm producer, displayed an average OD_{570} of 0.94 ± 0.13. In contrast, negative controls (blanks) showed an average OD_{570} of 0.16 ± 0.02.

Among the species of mastitis pathogens, *S. aureus* exhibited the highest biofilm production with an average OD_{570} of 0.24 ± 0.24, followed by *E. coli* (0.19 ± 0.22) and *S. chromogenes* (0.18 ± 0.17) (Fig 1a). All groups exhibited significant differences compared to

the positive control *S. aureus* O46 ($P < 0.01$), but no significant differences were observed among groups of species ($P > 0.99$).

Analysis of strain-specific biofilm formation indicated great variation among the strains tested. A subset comprising one-tenth of the strains (n=5) displayed no significant differences compared to the O46 control ($P > 0.05$) (Fig. 1b). These biofilm producers, ranging from weak to moderate, were distributed across various species groups. This group included two strains of *K. pneumoniae* (strains 158 and 160), two of *E. coli* (strains 219 and 227), *S. aureus* strain 186. Within this cohort, two strains were categorized as MBF (*K. pneumoniae* 158 and *E. coli* 227).

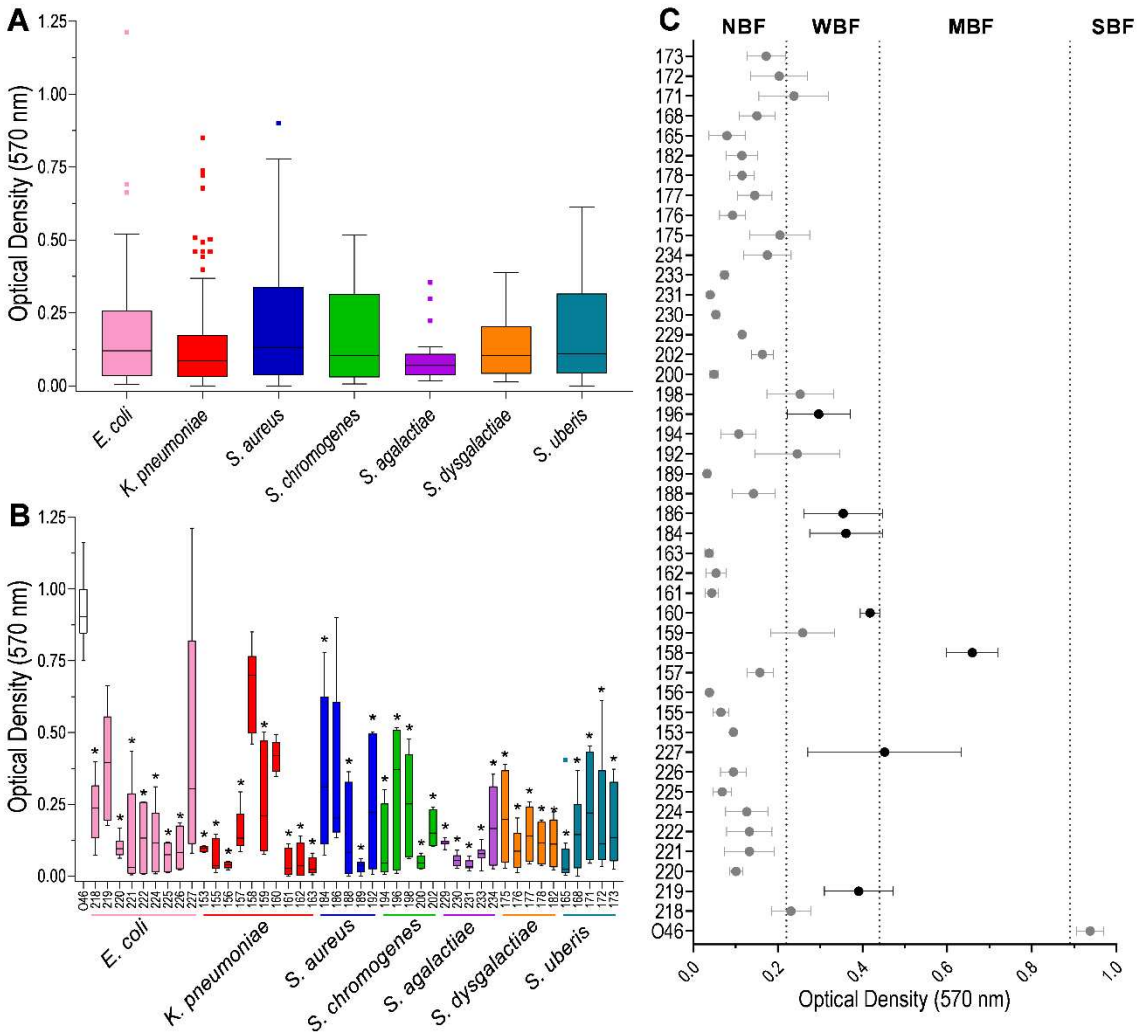

Figure 1: In vitro biofilm-forming capacity of mastitis-causing strains of *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcs chromogenes, Streptococcus*

agalactiae, Streptococcus dysgalactiae and *Streptococcus uberis* species. a) Biofilm-forming capacity of strains grouped by species ($P < 0.05$). Colored squares identify outliers detected by Tukey's method. b) Biofilm-forming capacity of strains categorized by species and color-coded as per the legend. Asterisks show a significant difference ($P < 0.05$) in biofilm formation compared to control (*S. aureus* O46). c) Classification as non-biofilm former (NBF), weak-biofilm former (WBF), moderate-biofilm former (MBF), and strong-biofilm former (SBF) according to Stepanovic *et al.* (2000). The highest biofilm formers strains are indicated by black dots.

Effect on bacterial growth

To assess the impact of lyn-1 on bacterial growth, the strains selected for biofilm formation were pooled and exposed to different concentrations of the peptide over 24 hours (Fig. 2A). A decrease in growth rate was observed when the concentration of lyn-1 was 32 µg/mL, but the area under the curve (AUC) was not different ($P = 0.32$) compared to the control (35.36 ± 1.36 for the control and 35 ± 1.01 for the 32 µg/mL treatment, Fig. 2B). However, the addition of lyn-1 at 64 µg/mL and 128 µg/mL dramatically reduced growth ($P < 0.01$), and the final OD_{600} averaged 0.19 ± 0.13 and 0.04 ± 0.03, respectively (Fig. 2A and B). These results indicate that lyn-1 could be an effective alternative for treating polymicrobial infections associated with bovine mastitis.

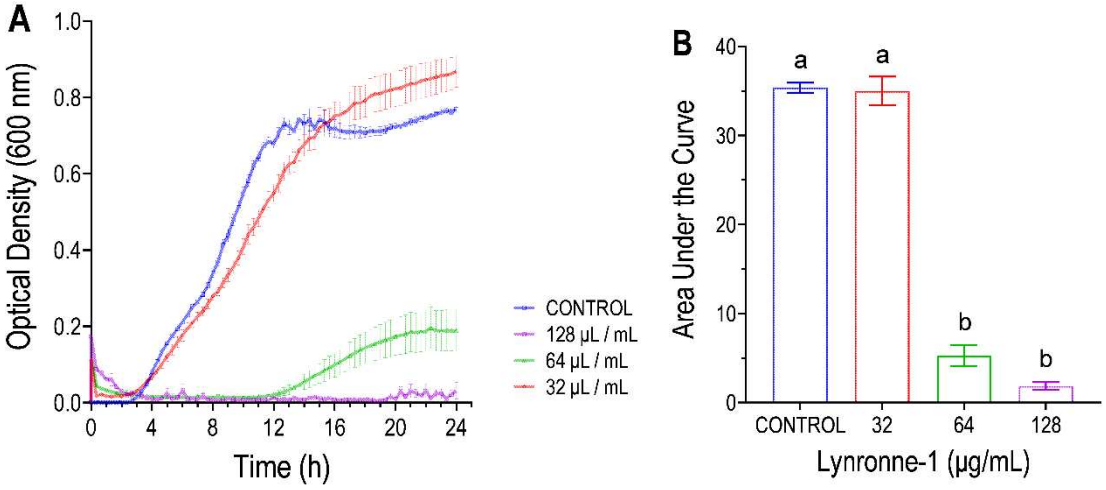

Figure 2: Effect of Lynronne-1 on the growth of pooled biofilm forming bacteria. Bars show the standard error of the mean. a) Growth curve of bacterial targets exposed to different concentrations of lyn-1 according to color-coded legend. b) Area under the curves. Different lowercase letters indicate statistical differences using the Tukey test ($P<0.05$).

Anti-biofilm activity

Lyn-1 exhibited a significant reduction in biofilm formation by 75% compared to the control ($P < 0.01$) (Supplementary Figure 1). Lyn-1 exhibited significant efficacy in reducing the pre-established biofilm, particularly against *S. aureus* strain 184 ($P < 0.01$). While its impact on established biofilms of other strains was not as pronounced, Lyn-1 also attenuated new biofilm formation by *S. chromog*enes 196 ($P < 0.05$). Moreover, Lyn-1 effectively prevented biofilm development in strains *E. coli* 227, *K. pneumoniae* 158 and 160, *S. aureus* 186, and the bacterial pool comprising all seven strains ($P < 0.05$).

Lyn-1 treatment did not reduce biofilm formation by *E. coli* 219 compared to baseline ($P > 0.05$). However, while untreated control exhibited a substantial increase of 142.31% in biofilm formation after 24 hours, lyn-1 prevented new biofilm formation by 46.14% (Fig. 3A). Baseline and control were significantly different ($P = 0.02$), but similar to the lyn-1 treatment ($P > 0.05$). For *E. coli* 227, classified as MBF, lyn-1 showed a numerically lower (58.80%) biofilm formation compared to baseline, but this difference was not statistically significant ($P = 0.20$) (Fig. 3B). Furthermore, the untreated control doubled the amount of biofilm produced (107.69% higher) after 24 hours ($P < 0.01$).

The untreated control of *K. pneumoniae* 158 (MBF) showed a 26.03% increase in biofilm formation after 24 hours of incubation, while lyn-1 caused a 22.95% reduction (Fig. 3C). Although there was a trend (ANOVA, $P = 0.07$), no significant differences were observed between treatments (Tukey, $P > 0.05$). *K. pneumoniae* 160 showed an increase of 155.67% in biofilm compared to the baseline, while cultures treated with lyn-1 reduced biofilm formation by 63.28% (Fig. 3D). The untreated control was statistically different from the other groups ($P < 0.05$), but there was no significant difference between the baseline and the treatment ($P = 0.37$).

For *S. aureus* 184, the control group increased biofilm by 58 % (Fig. 3E). In contrast, lyn-1 induced a reduction of biofilm formation by 82.21% compared to the baseline. All groups

were statistically different from one another ($P < 0.01$). Lyn-1 also caused a 70.92% reduction in biofilm formation by *S. aureus* 186 compared to the baseline (Fig. 3F), although this difference was not statistically significant ($P = 0.30$). However, the cultures in the control group displayed a significant increase of 224.92% in biofilm formation after incubation for 24 hours ($P < 0.01$). In addition, biofilm formation by *S. chromogenes* 196 was 208.15% higher than the baseline, and lyn-1 only reduced biofilm formation in comparison to the control (Fig. 3G). All groups were statistically different from one another ($P < 0.05$).

Biofilm formation in the bacterial pool containing all seven strains was generally lower for the control group compared to individual strains, with only a 2.9% increase from the baseline. Although lyn-1, on average, produced 33.18% less biofilm, no differences were detected between groups ($P > 0.05$).

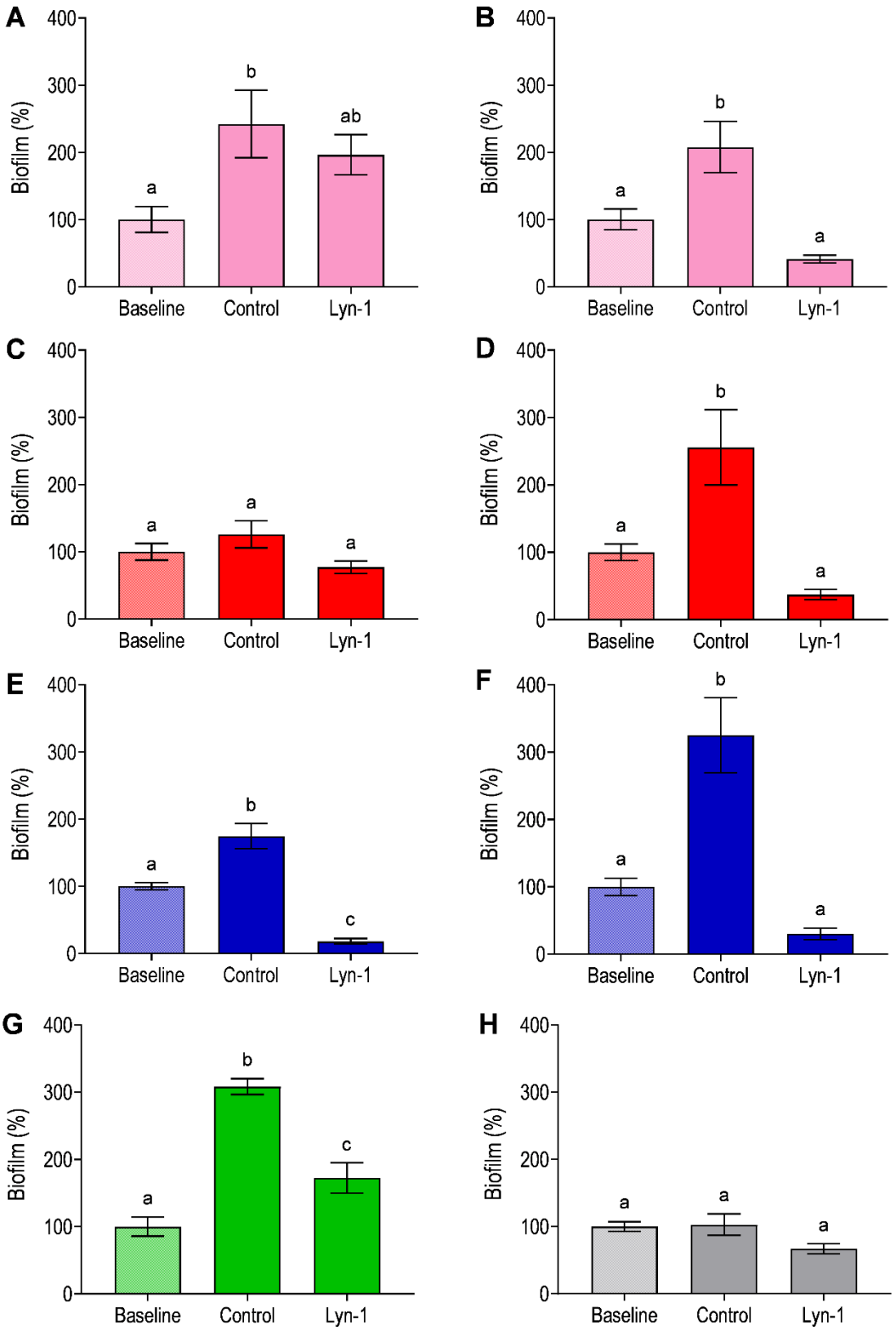

Figure 3: Effect of lynronne-1 in disrupting established bacterial biofilms. Different lowercase letters indicate statistical difference using the Tukey test ($P<0.05$). a) Effect on biofilm of

Escherichia. coli 219. b) Effect on biofilm of *E. coli* 227. c) Effect on biofilm of *Klebsiella pneumoniae* 158. d) Effect on biofilm of *K. pneumoniae* 160. e) Effect on biofilm of *Staphylococcus aureus* 184. f) Effect on biofilm of *S. aureus* 186. g) Effect on biofilm of *Staphylococcus chromogenes* 196. h) Effect on biofilm of a pool of all seven biofilm-forming targets.

Prevention of bacterial adhesion and invasion

The effectiveness of Lyn-1 in preventing bacterial infections on MAC-T cells was assessed to determine its potential as a preventive or therapeutic measure for bovine mastitis. Analysis of the mean log10 values of CFU/mL across seven strains revealed a significant decrease in adhesion and invasion rates at both infection concentrations ($P < 0.01$). At a 1:1 ratio of infection, Lyn-1 led to an average reduction of 4 log cycles, which corresponds to approximately 99.99% reduction in adhered bacteria. Regarding invasive bacteria, a reduction of 2.2 log cycles was observed (approximately 99.38%). At a 1:100 ratio of infection, Lyn-1 resulted in an average reduction of approximately 1.9 log cycles, equivalent to 98.92%, in adhered bacteria. For invasive bacteria, a reduction of 1.6 log-cycles (~ 97.62%) was observed (see Fig. 4). Data categorized by strain is illustrated in Supplementary Figure 2.

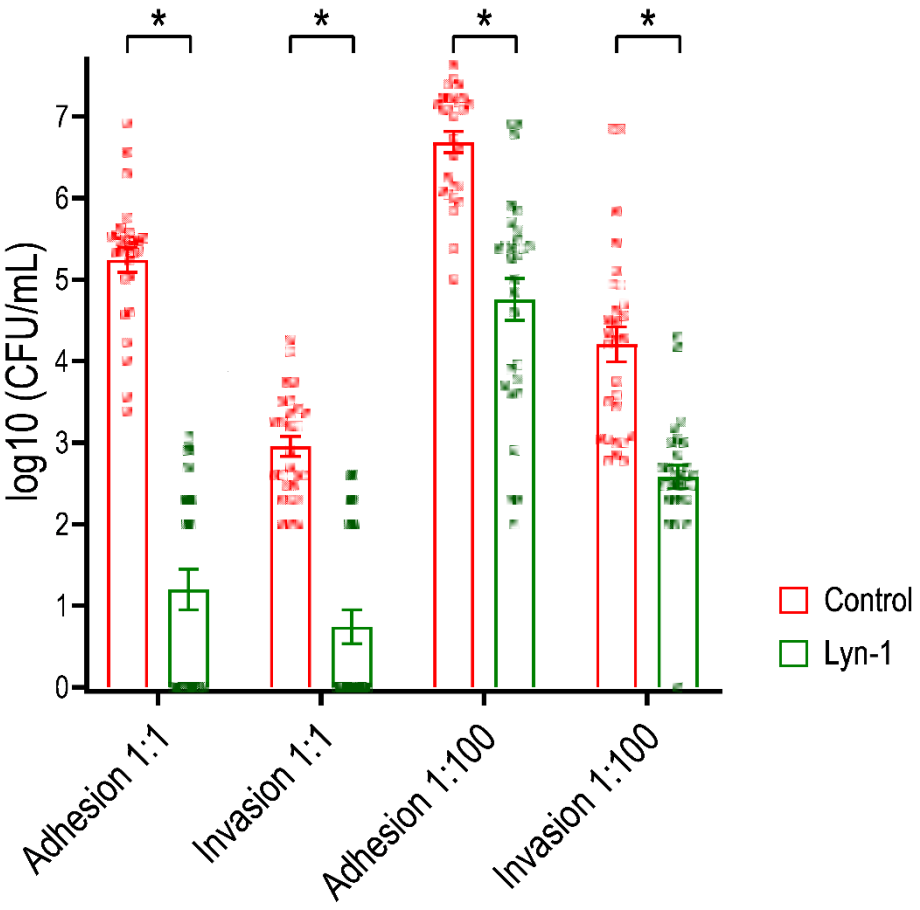

Figure 4: Boxplot showing the capacity of Lynronne-1 to prevent infection caused by mastitis-causing pathogens. Bars show the standard error of the mean. Colored squares identify observations. Asterisks show a significant difference ($P < 0.05$) in colony formation between treatment (green) and control (red), assessed by multiple t-tests.

Viability of Bacterial Populations

The viability of adhered *Staphylococcus aureus* strain 184 and infected MAC-T cells post-treatment with lyn-1 was assessed using fluorescence staining to evaluate its efficacy against bacteria and safety for eukaryotic cells. Approximately 10^5 MAC-T cells were infected with 10^7 CFU of *S. aureus* 184, resulting in a multiplicity of infection (MOI) of 1:100. The treated group received Lyn-1 at a concentration corresponding to the MIC of 128 µl/ml. In the untreated control group (Fig. 5a and 5c), fluorescence microscopy at 1000x magnification revealed numerous green dots indicative of live *S. aureus* bacteria attached to one MAC-T cell. At a lower magnification of 400x (Fig. 5c), a dense bacterial load of live *S. aureus* cells

(depicted as smaller green dots) was observed surrounding dead MAC-T cells (depicted as larger red dots). Upon application of lyn-1 treatment (Fig. 5b and 5d), significant changes were observed. At 1000x magnification (Fig. 5b), individual MAC-T cells were observed surrounded by a reduced *S. aureus* cell density and a larger number of dead bacteria. At 400x magnification (Fig. 5d), the treated samples exhibited a higher number of live MAC-T cells surrounded by dead *S. aureus* bacteria.

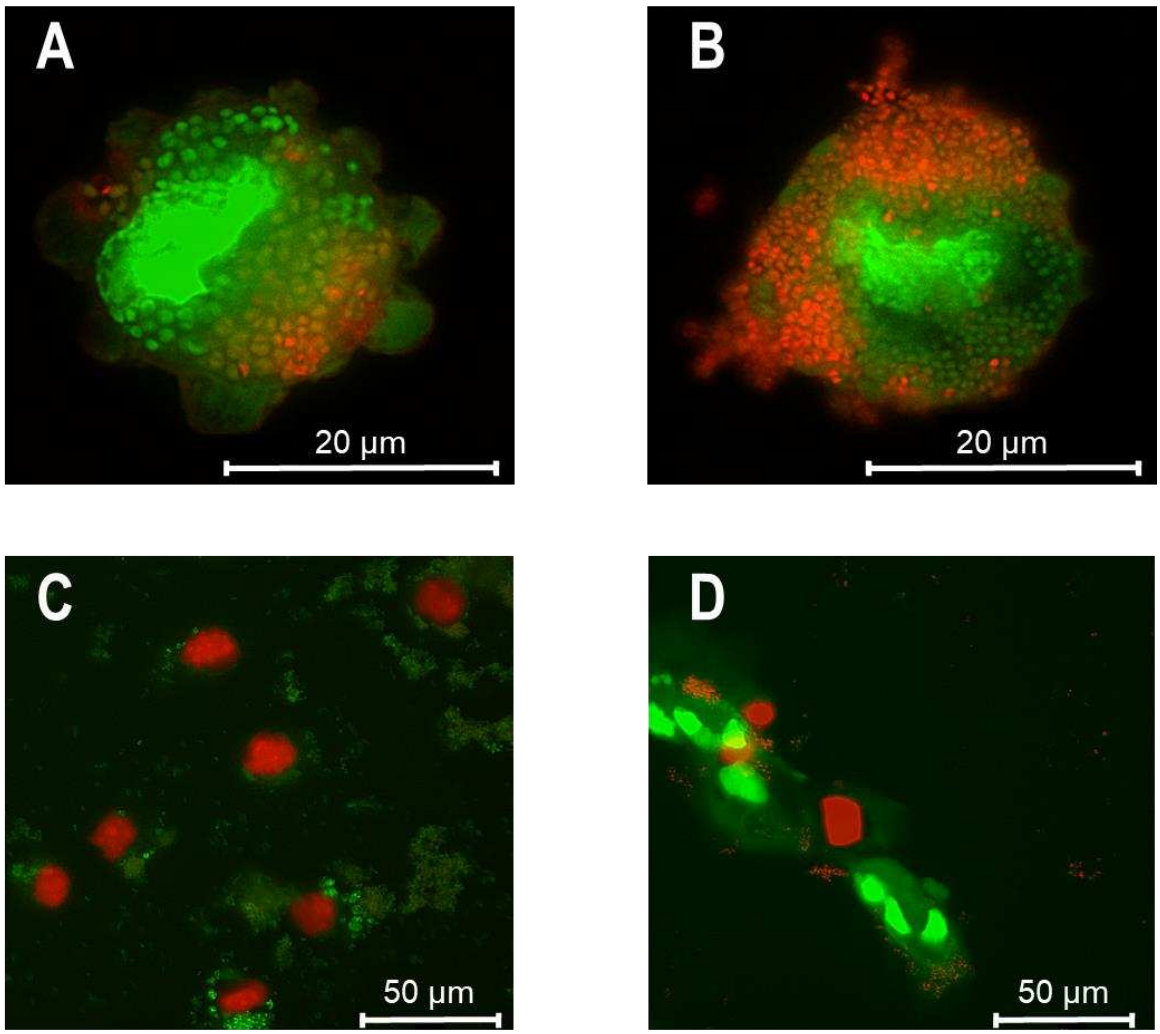

Figure 5: Photomicrographs from fluorescence microscopy of MAC-T cells infected by *Staphylococcus aureus* strain 184 at MOI 1:100. Live cells are indicated in green, while dead cells are shown in red. a) Untreated control MAC-T cell with live *S. aureus* (depicted as smaller dots) adhered to the cell surface, at 1000x magnification. b) Treated MAC-T cell displaying dead *S. aureus* cells attached at 1000x magnification. c) Untreated control group exhibiting multiple dead MAC-T cells surrounded by live *S. aureus* cells at 400x magnification. d) Treated

group with increased MAC-T cell viability and reduced *S. aureus* cell density compared to the control at 400x magnification.

DISCUSSION

Mastitis, a prevalent inflammatory disease affecting dairy cows, poses significant threats to both animal health and agricultural economics. The virulence of mastitis pathogens and their antibiotic resistance are intricately linked to the formation of biofilms. In this study, we delve into the influence of the antimicrobial peptide Lynronne-1, identified in the rumen microbiome through functional metagenomics, on established biofilms formed by major mastitis pathogens. From our collection, we selected seven highest biofilm producers, including two moderate and five weak formers, as targets. The impact of Lyn-1 on the growth of these strains was assessed at various concentrations, confirming a previously established MIC value of 128 µg/mL. Notably, lyn-1 demonstrated efficacy in preventing new biofilm formation in four strains and reduced new biofilm formation in two strains. Most significantly, lynronne-1 decreased the established biofilm by 82% in the case of *Staphylococcus aureus* 184. Furthermore, we evaluated the effectiveness of Lyn-1 against bacterial infections on MAC-T cells. Our findings reveal significant reductions in both adhered and invasive bacteria under two different multiplicity of infection conditions. Importantly, fluorescence staining suggested that Lyn-1 selectively targets bacteria while maintaining potential safety for eukaryotic cells, indicating its promise as a future treatment.

Biofilm formation by bacteria that penetrate the teat and adhere to mammary tissue has been linked to chronic and recurring mastitis infections (Melchior, Vaarkamp and Fink-Gremmels, 2006) and proposed as a factor contributing to multidrug resistance (MDR) in *E. coli* strains (Tyerman *et al.*, 2013; Gędas and Olszewska, 2020). Addressing biofilm prevention as an alternative to traditional antibacterial agents has emerged as a potential strategy for traditional antibacterial agents. In this context, lyn-1 shows promise, displaying anti-biofilm activity against 24-hour biofilms at concentrations of ≥2× MIC (32 µg/mL) against methicillin-resistant *S. aureus* ATCC 33591 (Oyama *et al.*, 2017). However, lyn-1 did not exhibit significant activity at 3× MIC against established biofilms of *Pseudomonas aeruginosa* PAO1 (96 µg/mL) and LES431 (24 µg/mL) (Mulkern *et al.*, 2022). These strains are recognized as strong biofilm formers (Davarzani *et al.*, 2021; Goodyear *et al.*, 2022; Leesombun *et al.*, 2023).

In our study, Lyn-1 at 1× MIC (128 µg/mL) demonstrated significant anti-biofilm activity against the bacterial pool, primarily preventing biofilm attachment. This effect may be attributed to bacterial growth inhibition. Lyn-1's ability to disrupt established biofilms was observed against the 48-hour biofilm of *S. aureus* 184 and prevented additional biofilm formation in *E. coli* 227 (MBF) and *K. pneumoniae* 160 and *S. aureus* 186, classified as WBFs. It also reduced additional biofilm formation in *E. coli* 219 and *S. chromogenes* 196. These findings suggest that Lyn-1 has potential against certain bacterial biofilms, influenced by dosage and specific bacterial strains, necessitating further research for optimization.

Furthermore, bacterial adherence and internalization play crucial roles in facilitating the delivery of virulence factors and toxins, aiding nutrient acquisition, and evading the host immune system and antibiotics (Cozens and Read, 2012; de Souza Santos and Orth, 2014; Owrangi *et al.*, 2017). Adherence confers tissue tropism, enabling pathogenic bacteria to exploit suitable environments to meet their physiological and metabolic requirements. Lyn-1 reduced bacterial adhesion and internalization rates across different infection conditions while sparing eukaryotic cells, highlighting its potential as an effective treatment.

CONCLUSION

In conclusion, lyn-1's ability to inhibit biofilm formation, and reduce bacterial adherence and internalization, combined with its safety profile for eukaryotic cells, underscores its potential as a therapeutic or prophylactic agent for bovine mastitis. Further studies are necessary to evaluate Lyn-1's safety and efficacy when administered as an intramammary infusion for bovine mastitis prevention or treatment *in vivo*

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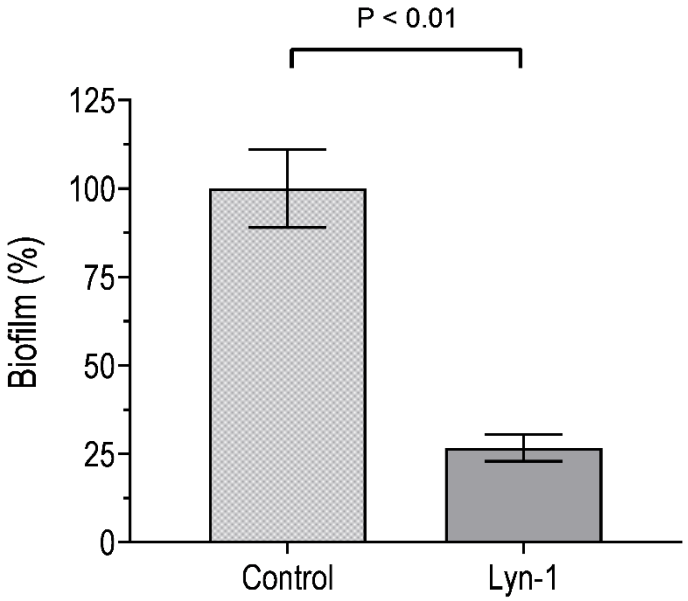
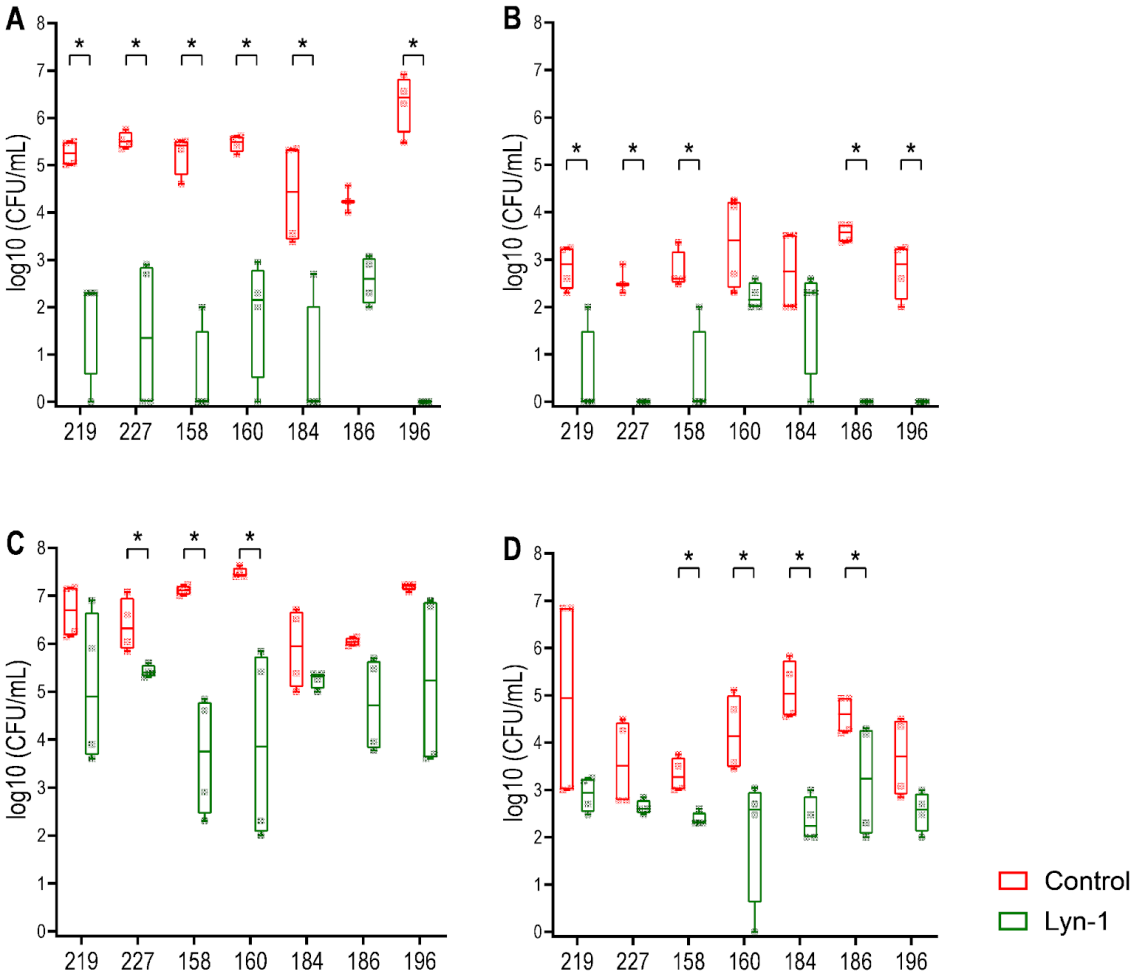
SUPPLEMENTARY MATERIAL



Supplementary Figure 1: Bar plot demonstrating the capacity of Lynronne-1 to prevent biofilm attachment of mastitis-causing strains capable of biofilm formation. The P-value indicates significant differences between groups, assessed using the Mann-Whitney test.



Supplementary Figure 2: Boxplot demonstrating the capacity of Lynronne-1 to prevent infection caused by mastitis-causing strains. Asterisks show a significant difference ($P < 0.05$) in colony formation between treatment (green) and control (red), assessed by multiple unpaired t-tests. a) Adhesion at MOI 1:1. b) Invasion at MOI 1:1. c) Adhesion at MOI 1:100. d) Invasion at MOI 1:100.

Supplementary Table 1: Biofilm formers strains ranked by average OD_{570}.

Species	Strain	Classif.	Mean	SD[1]	SEM[2]	CV[3]	P-value[4]
K. pneumoniae	158	MBF	0.66	0.15	0.06	0.22	0.71
E. coli	227	MBF	0.45	0.45	0.18	0.98	0.08
K. pneumoniae	160	WBF	0.42	0.06	0.02	0.13	0.34
E. coli	219	WBF	0.39	0.20	0.08	0.51	0.22
S. aureus	184	WBF	0.36	0.26	0.09	0.72	0.03
S. aureus	186	WBF	0.35	0.28	0.09	0.79	0.06
S. chromogenes	196	WBF	0.30	0.22	0.07	0.75	<0.01
K. pneumoniae	159	WBF	0.26	0.19	0.08	0.72	0.02
S. chromogenes	198	WBF	0.25	0.19	0.08	0.77	0.01
S. aureus	192	WBF	0.25	0.25	0.10	1.01	<0.01
S. uberis	171	WBF	0.24	0.20	0.08	0.85	<0.01
E. coli	218	WBF	0.23	0.11	0.05	0.49	0.03

[1] Std. Deviation; [2] Std. Error of Mean; [3] Coefficient of variation.

[4] Determined by Dunn's test against positive control *S. aureus.*

CHAPTER 3

EFFICACY OF ANTIMICROBIAL PEPTIDE-INFUSED HYDROGEL TREATMENT FOR SUBCLINICAL MASTITIS IN LACTATING DAIRY COWS

ABSTRACT

Bovine mastitis is the most prevalent and costly disease in the dairy sector and represents the primary reason for antibiotic usage on dairy farms. With increasing concerns regarding antibiotic resistance, there is a pressing need for alternative treatments. Antimicrobial peptides (AMPs) have emerged as promising candidates due to their unique properties. Here, we investigated the efficacy of Lynronne-1 (Lyn-1) as an intramammary treatment for mastitis in Holstein cows with high somatic cell counts (SCC). The experiment was conducted at the Dairy Forage Research Center Farm in Prairie Du Sac, WI, USA. Multiparous cows with high SCC (n = 26) were enrolled in the study and paired based on SCC values. Cows were randomly assigned to receive either Lyn-1 (2.24 mg/ml, 70x MIC) in 2.5% chitosan hydrogel or a commercial antibiotic, AMOXI-MAST® (6.25 mg/mL amoxicillin). Quarters with similar scores in the California Mastitis Test were selected for treatment. Treatments were administered post-milking over four sessions, skipping the third, following standard farm protocols. Milk samples were collected before each milking on days 0, 1, 2, 5, 8, and 15 for SCC, total bacterial count (TBC), and composition analyses. Cows enrolled in the trial showed quarters with very high somatic cell counts indicating persistent inflammatory responses in the udder. The chitosan hydrogel containing Lyn-1 induced a transient increase in SCC, potentially indicative of an immune response. Baseline values were comparable between groups, with no significant differences (mixed-effects analysis, $P > 0.05$) observed during the study. Additionally, no antimicrobial residues were detected in milk post-treatment, highlighting the safety of Lyn-1. Only one case of mastitis was reported in the antibiotic-treated group. Our findings suggest that Lyn-1 holds promise as an effective and safe alternative for subclinical mastitis treatment in dairy cows.

Keywords: Antimicrobial peptide, bovine mastitis, chitosan hydrogel, alternative therapy.

INTRODUCTION

Bovine mastitis remains a significant economic burden in the dairy industry, with U.S. dairy producers alone facing estimated losses of $2 billion annually (Cobirka, Tancin and Slama, 2020). Estimates indicate that the prevalence of subclinical mastitis averages 42%, while clinical mastitis stands at 15% in dairy herds worldwide (Krishnamoorthy *et al.*, 2021). These conditions contribute to decreased milk productivity, increased handling costs, and reduced production yields in dairy farming (Rollin, Dhuyvetter and Overton, 2015). Although the primary treatment approach involves intramammary infusion of antibiotics, the rise in antibiotic resistance among mastitis pathogens has compromised the efficacy of clinically used antibiotics (Singh *et al.*, 2018; Abdi *et al.*, 2021), driving the need for alternative strategies (Ajose *et al.*, 2022).

Antimicrobial peptides (AMPs) have garnered attention for their potential in mastitis treatment, offering advantages such as high potency, low cytotoxicity, stability, and a reduced likelihood of resistance development (Hancock and Sahl, 2006; Yu *et al.*, 2018). Previous studies, such as that by Cao *et al.* (2007), have investigated the efficacy of AMPs like nisin in treating mastitis infections induced experimentally with various pathogens. Intramammary infusion of nisin at a dose of 2,500,000 IU demonstrated a clinical cure rate comparable to 0.8 g gentamicin (90.2% vs. 91.1%, respectively), highlighting its potential as an alternative treatment. Moreover, intramammary nisin treatment in early lactation showed economically beneficial outcomes (average of $19) for both multiparous and primiparous cows (Rodriguez *et al.*, 2024).

However, the efficacy of nisin is limited to Gram-positive bacteria (Helander and Mattila-Sandholm, 2000), posing a challenge with the evolving distribution of mastitis-causing pathogens, particularly in developed countries, with a shift from contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae* to environmental pathogens like environmental streptococci, *Klebsiella* spp., and *Escherichia coli* (Zadoks and Fitzpatrick, 2009). Gram-negative bacteria, such as *E. coli* and *K. pneumoniae*, are increasingly implicated in mastitis cases, resulting in higher costs compared to Gram-positive counterparts (Fu *et al.*, 2022). Therefore, alternative treatments must target a broad spectrum of pathogens.

Addressing this need, lynronne-1 (lyn-1), an antimicrobial peptide, has shown promise in controlling the proliferation of various bacterial pathogens (Oyama *et al.*, 2017). Lyn-1

exhibits potent activity against a spectrum of mastitis pathogens, including *E. coli, K. pneumoniae, S. aureus, Staphylococcus chromogenes, S. agalactiae, Streptococcus dysgalactiae*, and *Streptococcus uberis*. Additionally, lyn-1 demonstrates low cytotoxicity and effectively prevents bacterial infection in epithelial bovine mammary alveolar cells, underscoring its potential as a mastitis treatment.

Moreover, chitosan hydrogels have been extensively studied as a natural and efficient drug carrier system, due to their biodegradability, biocompatibility, non-toxicity, and antimicrobial properties (Moran *et al.*, 2018; Li *et al.*, 2021; Nguyen *et al.*, 2023). It has also been demonstrated that chitosan hydrogels serve as an effective carrier for AMPs (Rezaei *et al.*, 2020). Therefore, this study aimed to evaluate a lyn-1-based chitosan hydrogel formulation for intramammary infusion in mastitis treatment on a research dairy farm. By leveraging the synergistic effects of lyn-1 and chitosan hydrogel, we aim to provide a novel and effective alternative to conventional antibiotic therapies for mastitis control, addressing the evolving challenges posed by antimicrobial resistance and changing pathogen distribution in dairy farming.

MATERIAL AND METHODS

The study was conducted on the Dairy Forage Research Center Farm in Prairie Du Sac, WI, USA. The experimental procedures used in this research were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Agricultural and Life Sciences of the University of Wisconsin - Madison, USA (protocol A006601-A01).

AMP-Based Formulation

The intramammary formulation was prepared under aseptic conditions. Lyn-1, at a concentration of 2.24 mg/ml (70 times the minimal inhibitory concentration), was diluted in 2.5% (w/v) chitosan hydrogel. High molecular weight Chitosan (Sigma-Aldrich, San Luis, EUA) was dissolved in sterile ultrapure water, followed by the addition of sterile acetic acid (Sigma-Aldrich, San Luis, USA) to achieve a final concentration of 0.5% for gel formation. To enhance peptide solubility, a sterile solution of surfactant Tween 80 (Sigma-Aldrich, San Luis, USA) was added at a final concentration of 1%. Following preparation, the formulation was

distributed into sterile syringes, each containing 10 mL, and stored under refrigeration until the scheduled application.

Selection of cows

A total of 26 Holstein lactating cows, distributed across various lactation stages (Supplementary Table 1), were selected based on recent somatic cell count (SCC) records. The cow's diet consisted of 2.5% (0.7 kg) lactose, 9.1% (2.5 kg) canola meal, 7.7% (2.1 kg) roasted soybeans, 21.1% (5.8 kg) high-moisture corn, 29.9% (8.2 kg) alfalfa silage, and 29.7% (8.2 kg) corn silage. Two quarters from each animal were selected as experimental units for treatment based on the California Mastitis Test (CMT) scores. Quarters with similar CMT scores were grouped together and chosen for treatment, ensuring comparable initial SCC values between control and treatment groups. The cows were paired based on SCC values and randomly assigned to either the control or the treatment group. Two cows, one from each group, received treatment on only one quarter, due to the low CMT scores observed in the remaining three quarters.

Treatments administration

Prior to the main trial, an irritancy assessment was conducted using four healthy lactating cows to evaluate potential adverse effects in the udder. These cows received the intramammary chitosan hydrogel formulation post-milking across four sessions, excluding the third, in line with the anticipated trial protocol. Vital signs, including heart rate, respiratory rate, and rectal temperature, were recorded both before and 2 hours after application to monitor for any adverse reactions. Additionally, samples were collected to determine somatic cell count (SCC).

Cows were randomly assigned to either the control (antibiotic-treated) or treatment (AMP-hydrogel) groups. The control group comprised 13 cows and 24 quarters, receiving an intramammary infusion of Amoxicillin (AMX) at a concentration of 6.25 mg/mL (Amoxi-Mast, Merck Animal Health, Millsboro, DE). Conversely, the treatment group, also consisting of 13 cows and 24 quarters, received an intramammary infusion of lyn-1 at a concentration of 2.24 mg/mL. Treatments were administered post-milking across four sessions, excluding the third, following established farm protocols for mastitis treatment. After milking, each selected teat received the designated treatment (AMP or AMX) with gentle massaging from the teat

apex to the barrel to ensure uniform distribution and absorption of the treatment solution. Standard post-milking disinfection procedures were then carried out.

Milk sampling procedure

Each selected quarter of the udder underwent meticulous cleaning and aseptic sampling as previously described (Metzger *et al.*, 2018). The udder was initially wiped with a dry cloth to eliminate visible dirt, followed by pre-milking sanitation using chlorine dioxide. Subsequently, three streams of milk were discarded, followed by application of iodine solution for a minimum of 30 seconds. The teats were then dried with sterile gauze. After discarding another three streams of milk, the selected teat was wiped with sterile gauze moistened with isopropanol solution, and milk samples were collected in sterile conical 50 mL tubes, with some tubes containing 2-bromo-2-nitropropane-1,3-diol for preservation of SCC. Regular mechanical milking was then initiated. Samples were collected before the first intramammary infusion, and subsequently before milking on days 1, 2, 5, 8, and 15 post-treatment initiation.

Milk analysis

Milk samples preserved with 2-bromo-2-nitropropane-1,3-diol were sent to a commercial laboratory (AgSource, Menominee, WI) for SCC quantification and examination of milk composition parameters, including butterfat, protein, and lactose. Non-preserved milk samples underwent total bacterial count (TBC) assessment and detection of antimicrobial residues in milk. TBC determination was conducted using the drop plate technique for bacterial enumeration as described by Herigstad *et al.* (Herigstad, Hamilton and Heersink, 2001). Each milk sample was diluted in a phosphate-buffered saline (PBS) solution, and 5 µL of each dilution was plated on Brain Heart Infusion (BHI) agar plates. The plates were then incubated for 12 hours at 37°C followed by colony enumeration to calculate the Colony Forming Units (CFU) per milliliter.

Milk production per animal was evaluated using farm system records (BoviSync, Fond du Lac, WI), which tracked daily milking data. To assess antimicrobial residues in milk, samples collected on day 5 following the completion of the antibiotic withdrawal period (Merck Animal Health, 2015) underwent qualitative detection using the resazurin reduction assay. Each milk sample was dispensed into sterile 96-well plates. An inoculum of *Escherichia coli* 226, which is highly susceptible to Lyn-1 (MIC=8µg/ml), was prepared in Mueller-Hinton broth and added to each well at an initial concentration of 1.5×10^5 CFU/mL. Milk samples

were tested using ratios 1:1, 1:2, 1:4, and 1:8 to bacterial inoculum. Following overnight incubation, the plates were treated with a 0.02% resazurin solution. Results were interpreted after one hour, with blue color indicating no bacterial growth, and pink color indicating bacterial growth.

Statistical analysis

Data normality was assessed using the Shapiro-Wilk test, and mixed-effects analysis was employed to evaluate significant differences between treatments over time for various milk quality parameters. Log transformation was applied to SCC and TBC values for statistical analysis. Fisher's exact test was used to assess disparities in antimicrobial residues in milk. Significance was determined for p-values < 0.05, with comparisons considered as trends when $0.05 <$ p-value < 0.10. One cow from AMX group developed mastitis in one of its quarters at the end of the trial, leading to its exclusion from the experiment for the last day (day 15).

RESULTS

Somatic cell count

The AMX and AMP groups showed high, but similar baseline somatic cell count (SCC) values (1.2×10^6 and 1.5×10^6 cells/mL, respectively). After treatment application, the AMP group exhibited a marked increase in SCC, peaking at three times the initial counts on day 1 before gradually decreasing (Fig. 1). In contrast, the AMX group showed a modest increase in SCC post-treatment, which reversed by day 2. By day 5, SCC values for both groups returned to baseline levels (Fig. 1). After 7 days, the AMP group reached the lowest SCC values (8.5×10^5 cells/mL) during the trial, while the AMX group peaked at 2.1×10^6 cells/mL by day 15. A significant treatment-time interaction ($P < 0.01$) was observed, but no significant difference was found between treatments ($P > 0.05$) (Fig. 1).

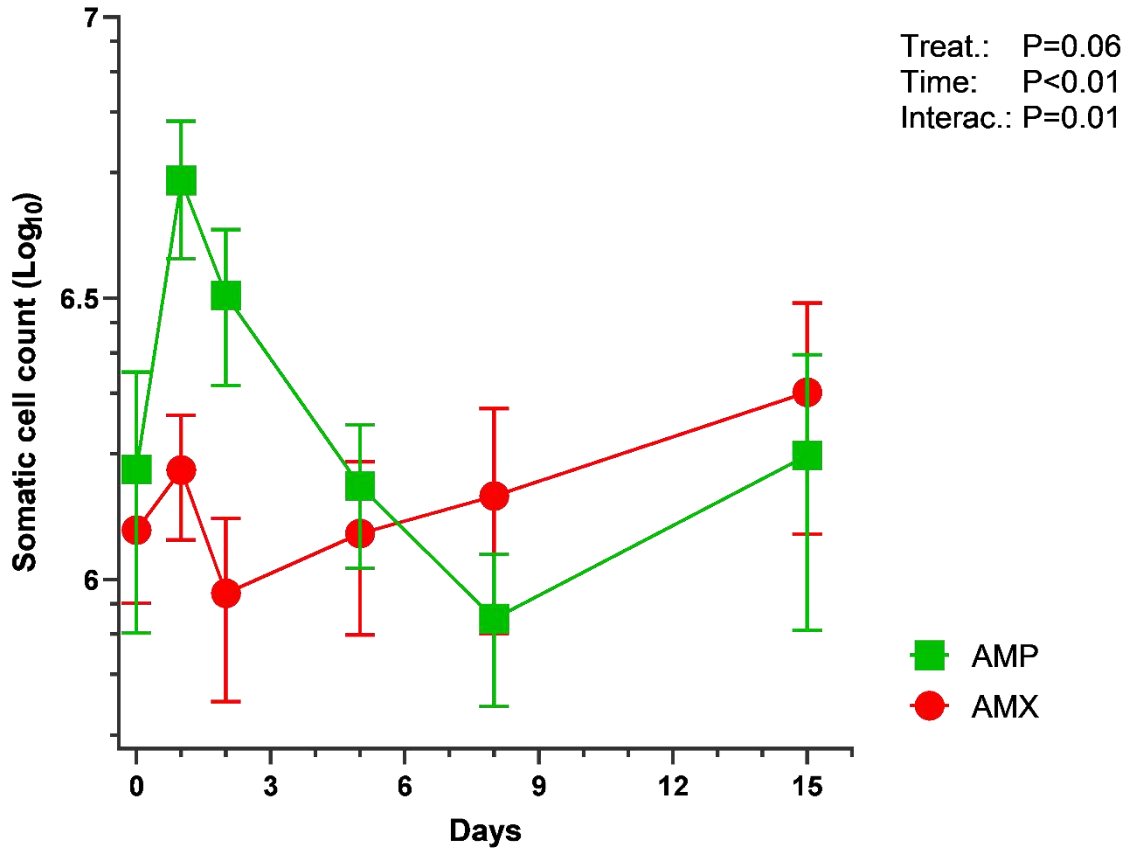


Figure 1: Effects of the Antimicrobial peptide-infused hydrogel-AMP (green squares) and Amoxicillin-AMX (red circles) on somatic cell counts. Bars show the standard error of the mean. No significant differences between treatments were found (Mixed-effects analysis, $P > 0.05$).

Total bacterial Count

The total bacterial count (TBC) at day 0 was comparable between groups, with values of 2.3 x 10^3 and 2.6 x 10^3 CFU/mL for the AMP and AMX groups, respectively (Fig. 2). Following treatment application, the AMP treatment exhibited a slight increase in TBC within 48 h, followed by a consistent decrease until the conclusion of the 15-day monitoring period, reaching a reduction of 48% compared to the initial population. Conversely, the antibiotic treatment showed a rapid decline in TBC after 5 days post-treatment, maintaining values around 0.9 x 10^3 CFU/mL at the end of the trial, despite a brief increase observed on day 8. Statistical analysis revealed a significant time effect ($P < 0.01$), while no significant treatment effects ($P=0.88$) or interaction between treatment and time ($P=0.97$) were observed. These

results indicate that both treatments had similar activities against total bacterial count over the duration of the study (Fig. 2).

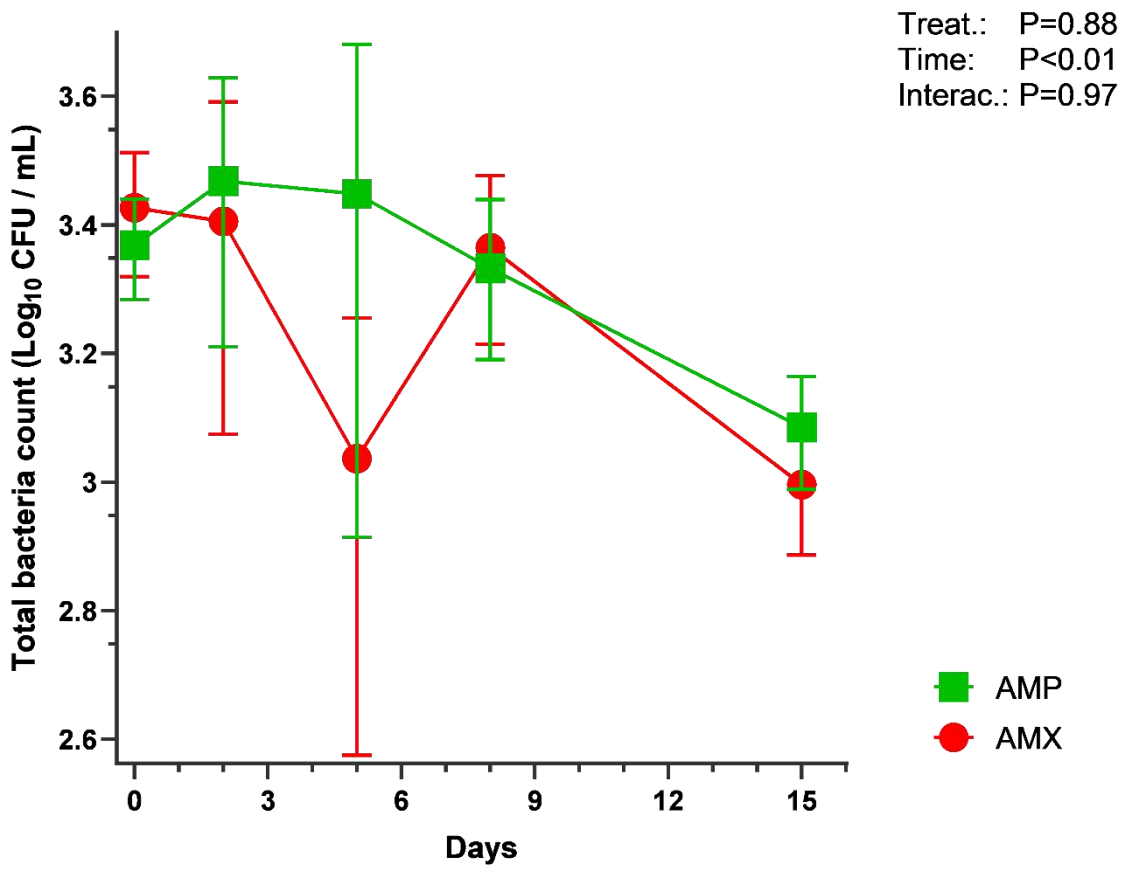


Figure 2: Effects of Antimicrobial peptide infused hydrogel – AMP (green squares) and Amoxicillin – AMX (red circles) on total bacterial count. Bars show the standard error of the mean. No significant differences between treatments were found (Mixed-effects analysis, $P > 0.05$).

Daily milk production

The AMP group exhibited consistently higher milk production until day 8, averaging approximately 7 liters more than the AMX group (86 and 79 L, respectively) during treatment application days (Fig. 3). Following treatments with AMX or AMP, both groups exhibited a slight decrease in milk production within 24 h, returning to normal levels the subsequent day. Peak production for both groups was observed on day 8 (88 and 82 L for the AMP and AMX groups, respectively). However, by day 15, the difference in milk production was 11 liters (84 and 73 L for the AMP and AMX groups, respectively), primarily due to a greater drop in milk

production among AMX-treated animals. Overall, no significant differences in milk production were observed between treatments (P > 0.05) (Fig. 3).

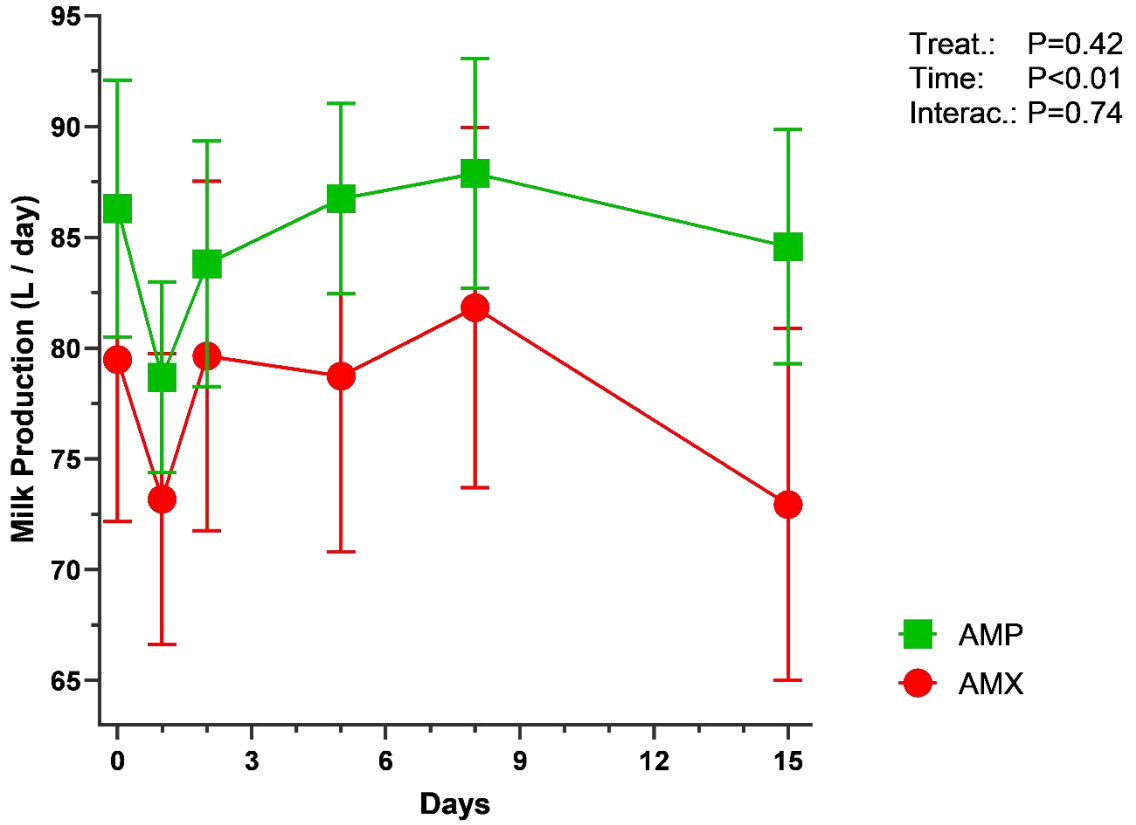


Figure 3: Daily production of cows treated with Antimicrobial peptide infused hydrogel – AMP (green squares) and Amoxicillin – AMX (red circles). Bars show the standard error of the mean. No significant differences between treatments were found (Mixed-effects analysis, P > 0.05).

Milk composition

The intramammary infusion of AMP or AMX induced changes in milk composition over time (P < 0.01) (Fig. 4). The effects on butterfat and protein (Fig. 4a and 4b) were similar between groups. Butterfat concentration peaked after 24 h post-infusion and decreased from day 2 to day 8. No significant differences in butterfat between treatments or interaction between treatments and time were observed (P > 0.05) (Fig. 4a). The AMX and AMP groups also showed similar protein levels at baseline (33 and 32.6 g/L, respectively) (P > 0.05). Similar to what was observed for butterfat, both groups experienced a sharp increase in milk protein levels on day 1 post-infusion. However, from day 2 onwards, the protein levels of the AMP group

remained numerically higher than those of the AMX group, around 34 g/L, until the end of the experiment. In contrast, after day 5, the protein levels of the AMX group continued to drop and reached 32.7 g/L by day 15. No significant differences in protein concentration between treatments or significant interactions between treatments and time were observed ($P > 0.05$) (Fig. 4b). The AMX and AMP groups had similar baseline lactose levels (45.8 and 45.3 g/L, respectively). However, the AMP group experienced a sharp decrease in lactose concentration within 24 h post-infusion compared to the AMX group ($P=0.05$) but returned to baseline levels by day 5. Conversely, the AMX group showed only a modest decrease in lactose concentration post-treatment. At day 8, the lactose levels of the AMP and AMX groups were similar ($P > 0.05$) and remained around 46 g/L until the end of the experiment (day 15). A significant treatment-time interaction ($P = 0.01$) was observed between treatments (Fig. 4c). All samples from both treatments displayed bacterial growth upon inoculation with the indicator strain *E. coli* 226, regardless of the milk concentration tested or treatment ($P > 0.05$). Detailed results for each parameter for individual animals can be found in the supplementary material.

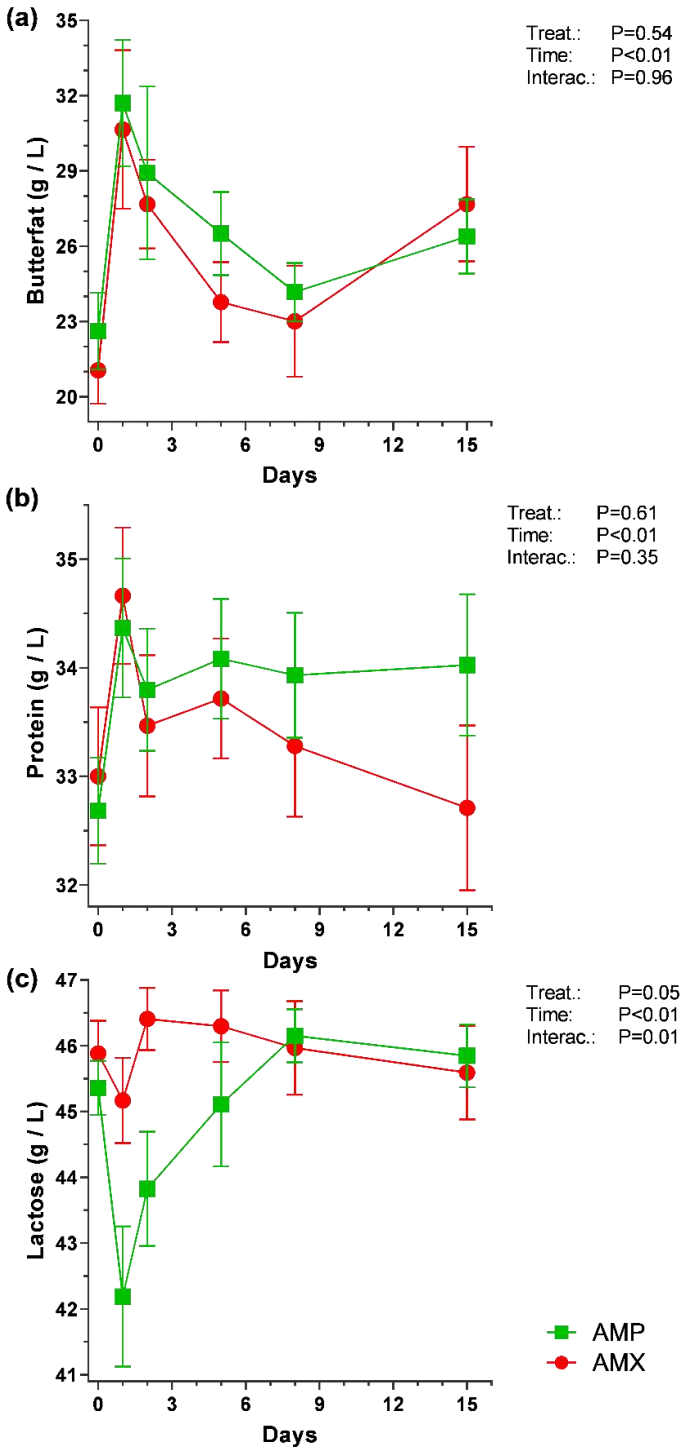


Figure 4: Milk composition of cows treated with Antimicrobial peptide infused hydrogel – AMP (green squares) and Amoxicillin – AMX (red circles). No significant differences between treatments were found (mixed-effects analysis, $P \geq 0.05$). a) Total milk butterfat. b) Total milk protein. c) Milk lactose.

DISCUSSION

Bovine mastitis continues to pose a significant challenge in the dairy sector, leading to considerable economic losses due to reduced milk production, treatment expenses, and the potential need to cull affected animals. Effective management of mastitis is crucial for safeguarding herd health and optimizing profitability (Heikkilä, Nousiainen and Pyörälä, 2012; Rollin, Dhuyvetter and Overton, 2015). The present study contributes valuable insights into the potential utility of lyn-1-embedded chitosan hydrogel as an alternative treatment for bovine mastitis, offering a promising avenue compared to conventional antibiotic therapy.

Although both treatments demonstrated comparable reductions in bacterial counts, differences in somatic cell count responses were observed between the AMP and AMX groups. Following lyn-1 treatment (AMP group), a transient increase in SCC was noted post-treatment, peaking on day 1 before gradually declining. This phenomenon could be attributed to the immune-enhancing properties of both antimicrobial peptides and chitosan hydrogel, as somatic cells in milk comprise both milk-producing cells and immune cells (Alhussien and Dang, 2018; Moran *et al.*, 2018; Li *et al.*, 2021; Darbandi *et al.*, 2022). It has been suggested that certain AMPs naturally produced by bacteria (bacteriocins), might exhibit a dual function, acting as bacterial inhibitors at high concentrations and as signaling compounds at lower concentrations, thereby attracting immune cells (Fajardo and Martínez, 2008). Thus, bacteriocins produced by probiotic strains could potentially function as quorum-sensing molecules or autoinducing peptides within the intestinal environment (Dobson *et al.*, 2012). Moreover, chitosan has been shown to stimulate macrophage activation and cytokine secretion from natural killer (NK) cells, primarily through phagocytosis-dependent mechanisms (Nishimura *et al.*, 1984; Shibata, Metzger and Myrvik, 1997). These effects have been observed to rely on phagocytosis (Li *et al.*, 2013). Previous research has elucidated a novel mechanism of chitosan action involving the activation of dendritic cells via type I interferon (IFN), fostering Th1-biased cellular immune responses (Carroll *et al.*, 2016). Notably, chitosan has gained recognition as a mucosal adjuvant, demonstrating efficacy in enhancing immune responses when administered with vaccines (Wang *et al.*, 2012).

Thus, we posit that the initial rise in SCC following AMP-embedded chitosan hydrogel application may be attributed to the immunomodulatory effects of its constituents, leading to an influx of immune cells. In contrast, the AMX-treated group initially experienced a decline in SCC post-treatment, which was subsequently reversed by day 2. This could be attributed to

the animals' history of high somatic cell counts and potential resistance to the regularly administered antibiotic. Furthermore, a negative correlation has been observed between somatic cell counts (SCC) and lactose concentration in milk, potentially explaining the observed low lactose levels during treatment, particularly during the peak SCC period in the hydrogel-treated (AMP) group (Wahyu Harjanti and Sambodho, 2020; Antanaitis *et al.*, 2021).

Despite the encouraging results, it is imperative to acknowledge certain limitations that may have influenced the observed outcomes. One limitation of this study was the selection of cows with high somatic cell count. Subclinical mastitis, characterized by persistent inflammation and tissue damage within the mammary gland, often leads to compromised udder health and reduced treatment response (Gonçalves *et al.*, 2020; Martins *et al.*, 2020). Such cows may exhibit altered mammary gland physiology and immune function, which could impact the effectiveness of antimicrobial treatments (Cheng and Han, 2020). Additionally, the immune response alterations associated with chronic mastitis may have influenced the study outcomes, given the pivotal role of the immune system in combating bacterial infections (Wellnitz and Bruckmaier, 2012). Furthermore, the study's duration may have limited the ability to assess the long-term efficacy of lyn-1-based hydrogel, considering the dynamic nature of mastitis and the potential variations in treatment response over time (Sharun *et al.*, 2021).

CONCLUSION

Our findings suggest that lyn-1-based hydrogel holds promise as an alternative treatment for subclinical bovine mastitis, demonstrating comparable outcomes to amoxicillin treatment. Further research is warranted to assess its long-term efficacy, safety, and potential integration into routine mastitis management practices. Additionally, economic analyses comparing the cost-effectiveness of lyn-1-based therapy to conventional antibiotic treatment would provide valuable insights for dairy producers considering the adoption of this novel approach.

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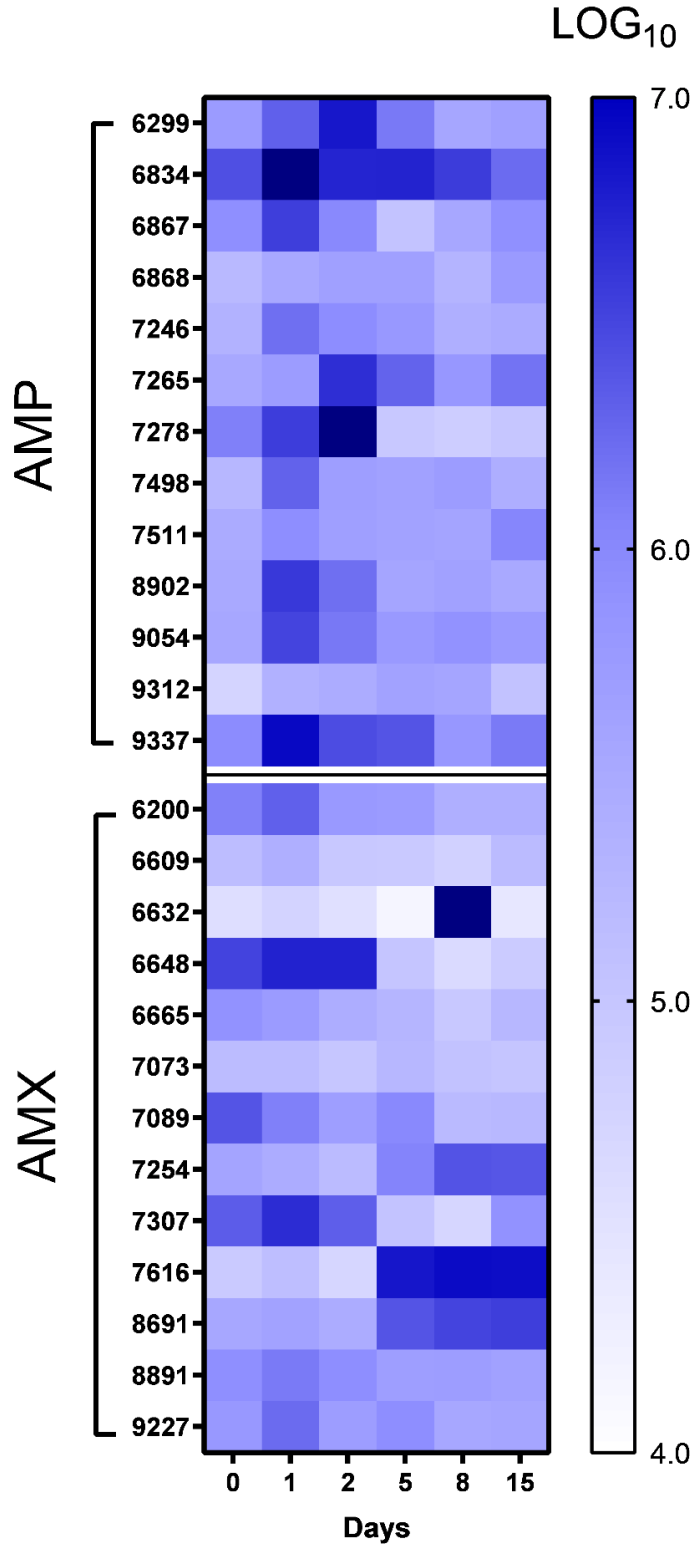
SUPPLEMENTARY MATERIAL

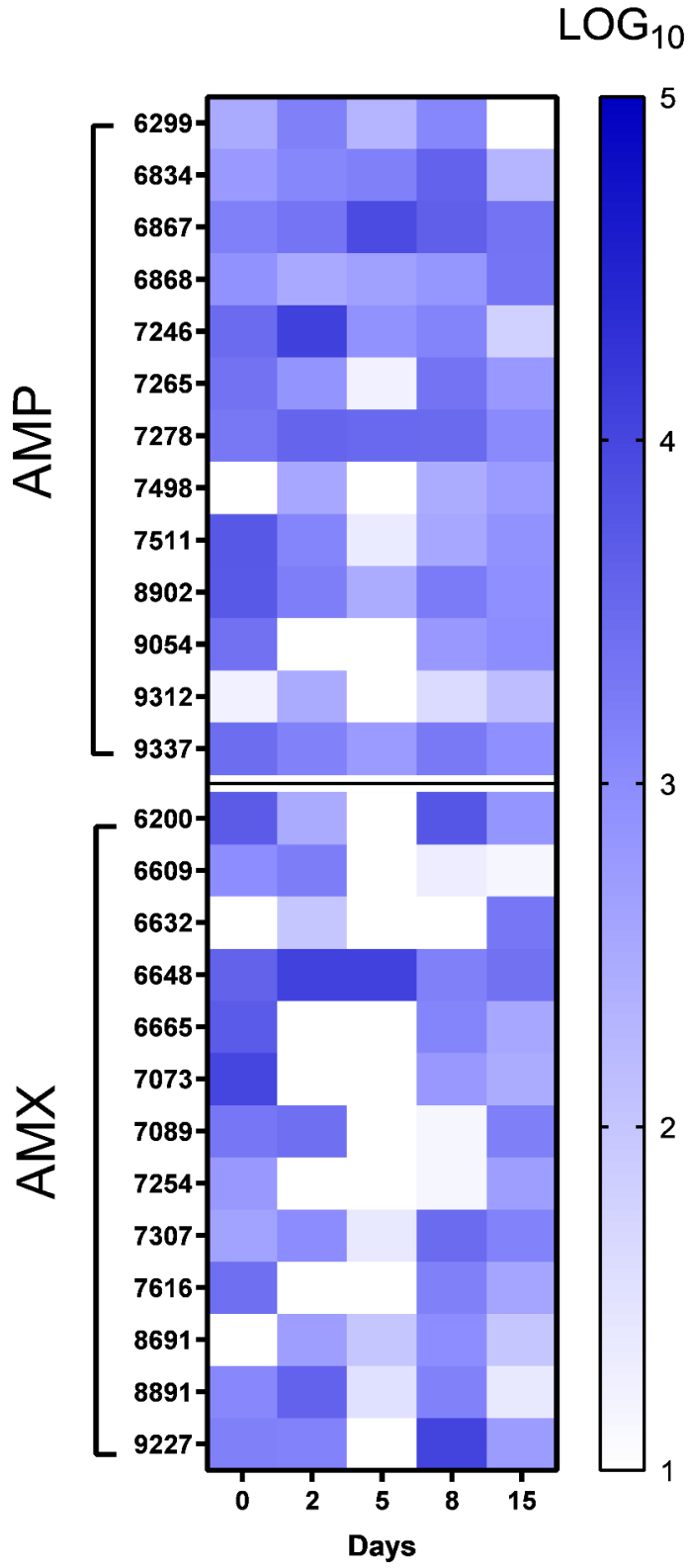
Supplemantary Table 1: Baseline characteristics of cows.

Animal	Age	Day of Parturition	Lactation	DIM[1]	Average Daily Milk Production
6200	7 yrs, 2 mo	21/09/2022	6	225	63
6299	6 yrs, 7 mo	02/08/2022	6	275	78
6609	6 yrs, 7 mo	07/02/2023	5	86	114
6632	6 yrs, 1 mo	08/09/2022	5	238	87
6648	6 yrs, 2 mo	02/11/2022	5	183	117
6665	6 yrs, 2 mo	21/12/2022	5	134	99
6834	5 yrs, 10 mo	23/11/2022	5	162	99
6867	5 yrs, 2 mo	16/07/2022	4	292	78
6868	5 yrs, 9 mo	03/02/2023	5	90	102
7073	4 yrs, 9 mo	22/08/2022	4	255	87
7089	4 yrs, 10 mo	12/10/2022	4	204	78
7246	4 yrs, 8 mo	28/11/2022	4	157	108
7254	4 yrs, 3 mo	26/07/2022	3	282	102
7265	4 yrs, 3 mo	27/08/2022	3	250	99
7278	4 yrs, 5 mo	21/10/2022	3	195	75
7307	3 yrs, 9 mo	26/04/2022	3	373	27
7498	2 yrs, 12 mo	10/07/2022	2	298	57
7511	2 yrs, 12 mo	30/08/2022	2	247	102
7616	3 yrs, 1 mo	29/03/2023	2	36	111
8691	5 yrs, 9 mo	22/07/2022	5	286	87
8891	5 yrs, 2 mo	04/07/2022	4	304	36
8902	5 yrs, 5 mo	28/09/2022	4	218	105
9054	4 yrs, 10 mo	09/09/2022	4	237	78
9227	4 yrs, 5 mo	04/09/2022	3	242	54
9312	4 yrs, 1 mo	27/08/2022	3	250	75
9337	3 yrs, 10 mo	04/06/2022	3	334	72

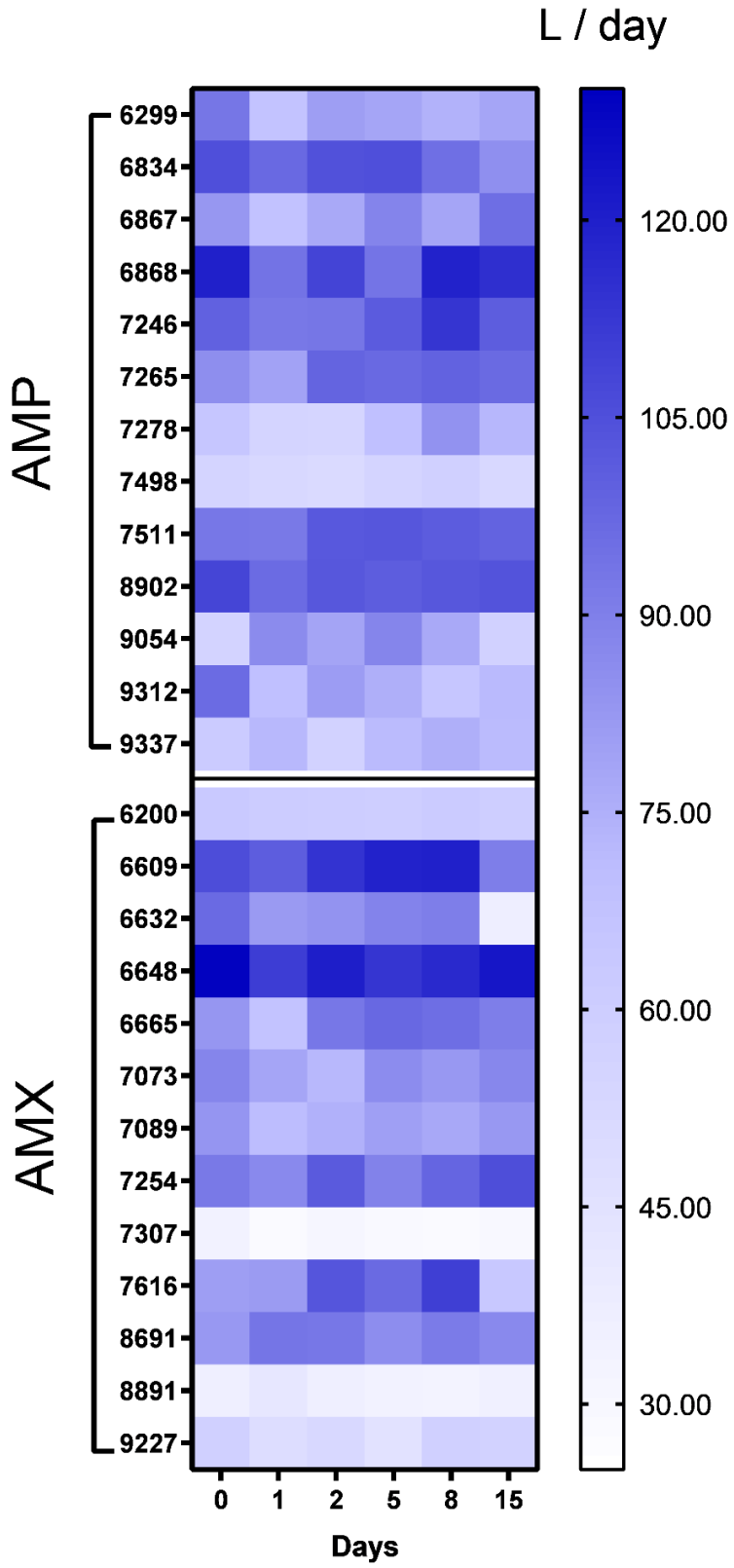
[1] Days in Milking;



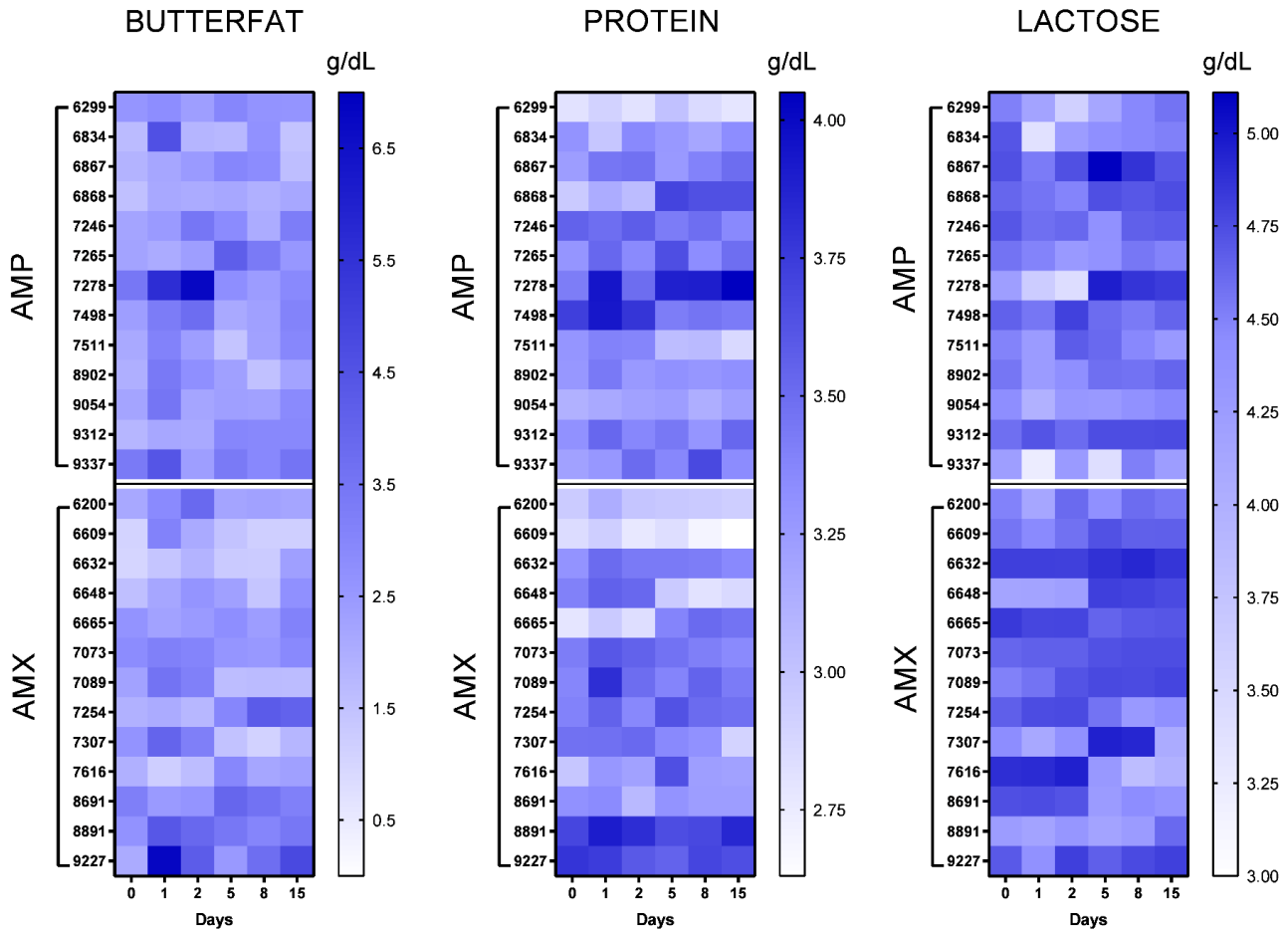
Supplementary Figure 1: Effects of Antimicrobial peptide infused hydrogel – AMP and Amoxicillin – AMX on somatic cell counts of individual cows over time.



Supplementary Figure 2: Effects of Antimicrobial peptide infused hydrogel – AMP and Amoxicillin – AMX on total bacterial counts of individual cows over time.



Supplementary Figure 3: Individual daily production of cows treated with Antimicrobial peptide infused hydrogel – AMP and Amoxicillin – AMX.



Supplementary Figure 3: Individual milk composition of cows treated with Antimicrobial peptide infused hydrogel – AMP and Amoxicillin – AMX.

GENERAL CONCLUSION

The potential of antimicrobial peptides (AMPs), particularly lynronne-1 (lyn-1), as effective alternatives to antibiotics in the management of bovine mastitis is underscored by the findings presented in this study. Potent antimicrobial activity against a broad spectrum of mastitis-causing pathogens was demonstrated by lyn-1 and EDTA through *in vitro* assessments, with synergistic effects and enhanced efficacy at lower concentrations observed in the Lyn-1-EDTA combination. Furthermore, lyn-1's ability to disrupt biofilms formed by mastitis pathogens was explored, with promising results in preventing biofilm attachment and reducing pre-formed biofilms revealed. Efficacy in preventing mastitis progression by reducing adhesion and invasion rates in epithelial bovine mammary alveolar cells was also exhibited by lyn-1. In a clinical context, comparable efficacy to conventional antibiotics in cows with high somatic cell counts was demonstrated by the intramammary treatment with lyn-1, highlighting its potential as a viable alternative for mastitis control. Importantly, the safety and sustainability of AMP-based treatments were underscored by the absence of detectable antimicrobial residues in milk samples. These findings collectively suggest that lynronne-1 and its combinatorial treatments hold promise as sustainable solutions for managing bovine mastitis, offering a pathway towards reducing antibiotic usage and mitigating antimicrobial resistance in the dairy sector. Future research should focus on further optimizing treatment protocols and evaluating their long-term efficacy and safety in clinical settings.

FINAL CONSIDERATIONS

This study presents compelling evidence regarding the efficacy of lynronne-1 and its synergistic interaction with EDTA, demonstrating strong bactericidal activity against mastitis-associated pathogens. Moreover, lyn-1 exhibits potential for inhibiting biofilm formation and reducing bacterial adherence and internalization, emphasizing its therapeutic and prophylactic utility. The safety profile of lyn-1, with minimal hemolytic activity and acceptable toxicity against eukaryotic cells, further supports its potential as a safe therapeutic agent for udder infections. Notably, the findings also highlight the need for continued investigation into the *in vivo* efficacy and safety of lyn-1, particularly when administered as an intramammary infusion. Overall, this work demonstrates how lyn-1-based therapies, including in combinations and intramammary formulations, offer promising alternatives to conventional antibiotics for managing bovine mastitis. This research sets the stage for future studies aimed at integrating AMP-based therapies into routine mastitis management protocols, potentially transforming the landscape of mastitis control with enhanced sustainability and improved outcomes for animal welfare, food safety, and public health.