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NISINA COMO ESTRATÉGIA ALTERNATIVA FRENTE À RESISTÊNCIA ANTIMICROBIANA - POTENCIAL TERAPÊUTICO E ABORDAGEM *IN VITRO* ENTRE MASTITE BOVINA E OTITE EXTERNA CANINA

REALEZA
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Dissertação apresentada ao Programa de Pós-Graduação em Saúde, Bem-Estar e Produção Animal Sustentável na Fronteira Sul da Universidade Federal da Fronteira Sul (UFFS), como requisito para obtenção do título de mestre.

Orientador: Profa. Dra. Dalila Moter Benvegnú

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RESUMO

A resistência antimicrobiana e a formação de biofilme se destacam entre os principais fatores associados à recorrência, cronicidade e falha terapêutica, colocando em destaque a necessidade da formulação de alternativas com potencial antimicrobiano e antibiofilme capazes de driblar a virulência e resistência antimicrobiana. Diante da limitação de novas opções farmacológicas, peptídeos antimicrobianos como a nisina - bacteriocina lantibiótica produzida por *Lactococcus lactis* - têm sido investigados como alternativas terapêuticas. Portanto, o presente estudo teve como objetivo avaliar a atividade antimicrobiana e antibiofilme e o perfil de toxicidade da nisina frente a bactérias isoladas de casos de otite externa canina e mastite bovina, visando explorar seu potencial no enfrentamento da RAM. No primeiro experimento, foram avaliados 34 isolados bacterianos provenientes de cães com diagnóstico de otite externa, incluindo *Corynebacterium* spp. e *Staphylococcus* spp. coagulase-negativa. Após identificação microbiológica e teste de suscetibilidade antimicrobiana, foram determinados a concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM), curva de crescimento e time-kill. Também foi avaliada a capacidade de formação de biofilme dos patógenos incluídos, bem como a capacidade de inibição e erradicação de biofilme pré-formado pela nisina. A toxicidade foi avaliada pelo ensaio de hemólise. A nisina apresentou atividade predominantemente bacteriostática e transitória e maior eficácia nas fases iniciais de formação de biofilme. As taxas de hemólise permaneceram abaixo do limiar considerado tóxico, indicando perfil de segurança favorável para aplicação clínica. No segundo estudo, foram avaliados 25 isolados de *Staphylococcus aureus* provenientes de casos de mastite bovina. Foram estimados o perfil de suscetibilidade antimicrobiana, capacidade de produção de biofilme, CIM, CBM e ensaios de inibição e erradicação do biofilme pré-formado. A nisina demonstrou atividade antimicrobiana frente aos isolados avaliados, com efeito dose-dependente e maior impacto na inibição da formação de biofilme em comparação à erradicação de biofilmes estabelecidos. Observou-se redução significativa da viabilidade bacteriana e da biomassa de biofilme em concentrações específicas. Em síntese, os resultados indicam que a nisina apresenta potencial como estratégia adjuvante no controle de infecções bacterianas associadas à otite externa canina e à mastite bovina, especialmente pela sua ação sobre fases iniciais de formação de biofilme. Apesar da atividade predominantemente bacteriostática e da eficácia reduzida frente a biofilmes maduros, o perfil de baixa toxicidade e a capacidade de interferir na persistência bacteriana reforçam sua relevância como alternativa promissora no contexto da RAM.

ABSTRACT

Antimicrobial resistance and biofilm formation stand out among the main factors associated with recurrence, chronicity, and therapeutic failure, highlighting the need for the development of alternatives with antimicrobial and antibiofilm potential capable of overcoming virulence and antimicrobial resistance. Given the limited availability of new pharmacological options, antimicrobial peptides such as nisin - a lantibiotic bacteriocin produced by *Lactococcus lactis* - have been investigated as therapeutic alternatives. Therefore, the present study aimed to evaluate the antimicrobial and antibiofilm activity, as well as the toxicity profile, of nisin against bacteria isolated from cases of canine otitis externa and bovine mastitis, in order to explore its potential in addressing AMR. In the first experiment, 34 bacterial isolates obtained from dogs diagnosed with otitis externa were evaluated, including *Corynebacterium* spp. and coagulase-negative *Staphylococcus* spp. Following microbiological identification and antimicrobial susceptibility testing, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), growth curve, and time-kill assays were determined. The biofilm-forming ability of the included pathogens was also assessed, as well as the capacity of nisin to inhibit and eradicate preformed biofilms. Toxicity was assessed using the hemolysis assay. Nisin exhibited predominantly bacteriostatic and transient activity, with greater efficacy during the early stages of biofilm formation. Hemolysis rates remained below the threshold considered toxic, indicating a favorable safety profile for clinical application. In the second project, 25 *Staphylococcus aureus* isolates obtained from cases of bovine mastitis were evaluated. Antimicrobial susceptibility profile, biofilm production capacity, MIC, MBC, and biofilm inhibition and eradication assays were performed. Nisin demonstrated antimicrobial activity against the evaluated isolates, with a dose-dependent effect and greater impact on inhibiting biofilm formation compared to the eradication of established biofilms. A significant reduction in bacterial viability and biofilm biomass was observed at specific concentrations. In summary, the results indicate that nisin shows potential as an adjuvant strategy for controlling bacterial infections associated with canine otitis externa and bovine mastitis, particularly due to its action on the early stages of biofilm formation. Despite its predominantly bacteriostatic activity and reduced efficacy against mature biofilms, its low toxicity profile and ability to interfere with bacterial persistence reinforce its relevance as a promising alternative in the context of antimicrobial resistance.

LISTA DE ILUSTRAÇÕES

Revisão bibliográfica:

Primeiro experimento:

Segundo experimento:

LISTA DE TABELAS

Primeiro experimento:

Segundo experimento:

Experimento adicional:

LISTA DE ABREVIATURAS E SIGLAS

ABC	Sistema de transporte
AMC	Amoxicilina com ácido clavulânico
AMP	Ampicilina
ANOVA	Análise de variância
ATCC	*American Type Culture Collection*
BHI	Caldo infusão cérebro coração
bMEC	Células epiteliais mamárias bovinas
BrCAST	*Brazilian Committee on Antimicrobial Susceptibility Testing*
CAT	Catalase
CD4+	*Cluster of Differentiation 4*
CD8+	*Cluster of Differentiation 8*
CLSI	*Clinical and Laboratory Standards Institute*
CLI	Clindamicina
CLO	Cloranfenicol
CN	Controle negativo
CP	Controle positivo
CIP	Ciprofloxacina
DNA	Ácido desoxirribonucleico
EM	Enrofloxacina
ERK/MAPK	*Extracellular Signal-Regulated Kinases / Mitogen-Activated Protein Kinases*
ESBL	Beta-lactamase de espectro estendido
FDA	*Food and Drug Administration*
GEN	Gentamicina
Gro- α	*Growth-Regulated Oncogene alpha*
HCl	Ácido clorídrico
H_2O	Água
IFN- γ	Interferon gama
IL	Interleucina
kDa	Quilodalton
LPS	Lipopolissacarídeo
M	Molar
MALDI-TOF	Ionização e dessorção a lazer assistida por matriz

MBC	Minimum Bactericidal Concentration
MBC50	Minimum Bactericidal Concentration required to inhibit 50% of isolates
MBC90	Minimum Bactericidal Concentration required to inhibit 90% of isolates
MBEC	Minimum Biofilm Eradication Concentration
MBIC	*Minimum Biofilm Inhibitory Concentration*
MCP-1	Proteína quimiotática de monócitos 1
MFX	Moxifloxacina
MHA	Ágar Mueller-Hinton
MHB	Caldo Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MIC50	Minimum Inhibitory Concentration required to inhibit 50% of isolates
MIC90	Minimum Inhibitory Concentration required to inhibit 90% of isolates
mm	Milímetros
MRSA	*Staphylococcus aureus* resistente à meticilina
MRSP	*Staphylococcus pseudintermedius* resistente à meticilina
mV	Milivolt.
NaCl	Cloreto de sódio
NET	Armadilha extracelular de neutrófilos
NF-κB	Fator nuclear kappa B
nm	Nanômetros
NRS	Sistema de resitência à nisina
NrsA	Proteína efetora de resistência à nisina
OD	Optical density
OXA	Oxacilina
PAMPs	Padrões moleculares associados a patógenos
PBS	Tampão fosfato salino
PCL	poli(ε-caprolactona)
PCR	Reação em cadeia da polimerase
PDR	*Pandrug resistant*
CMT	*California Mastitis Test*
PDI	Índice de polidispersão
PKA	Proteína quinase A
PKC	Proteína quinase C
PVA	Álcool polivinílico

RAM	Resistência antimicrobiana
rRNA	Ácido ribonucleico ribossomal
Rpm	Rotações por minuto
SISGEN	Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado
SOD	Superóxido dismutase
Sp.	Espécie
Spp.	Espécies
SUT	Sulfametoxazol + trimetoprima
TLR	Receptores do tipo Toll
TNF- α	Fator de necrose tumoral alfa
TSB	Caldo triptona de soja
UFC	Unidades formadoras de colônia
UI/mg	Unidades internacionais por miligrama
UI/mL	Unidades internacionais por mililitro
VRE	*Enterococcus* sp. resistente à vancomicina
WHO	*World Health Organization*

LISTA DE SÍMBOLOS

+	Positivo
-	Negativo/ausência
-	Indicação de intervalo (travessão)
±	Mais ou menos
/	Relação, associação ou divisão entre termos
Å	Ângstrom
%	Porcentagem
>	Maior que
<	Menor que
≈	Aproximadamente
$\log_{10}$	Logaritmo na base 10
α	Alfa
β	Beta
γ	Gama
κ	Kappa
®	Marca registrada
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SUMÁRIO

1 INTRODUÇÃO

A resistência antimicrobiana representa um dos principais entraves ao tratamento da infecções de origem bacteriana dentro da medicina e medicina veterinária, especialmente diante da limitação de novas opções farmacológicas no mercado e da prática ainda frequente da terapia empírica (Bourély *et al.,* 2019; Garcias *et al*., 2025; Wang *et al*., 2025). A escassez de novos antimicrobianos tem impulsionado a investigação de estratégias alternativas, como os peptídeos antimicrobianos, entre os quais se destaca a nisina, uma bacteriocina lantibiótica com atividade antimicrobiana consolidada, potencial antibiofilme e propriedades imunomoduladoras (Musiejuk; Kafarski, 2023; Oliveira *et al.,* 2024; Yuan *et al*., 2024).

A otite externa canina é uma das enfermidades mais prevalentes na clínica de pequenos animais, afetando cerca de 15% da população canina e apresentando caráter recorrente e multifatorial, com impacto significativo sobre o bem-estar animal, a qualidade de vida dos tutores e os custos terapêuticos (Perry *et al.*, 2017; Pye, 2018; Kwon *et al*., 2022; Li *et al.*, 2023). Nesse cenário, a elevada frequência de isolamentos bacterianos, a formação de biofilmes e a recorrência mesmo após tratamento adequado reforçam a complexidade do manejo clínico da doença e sua relevância no contexto da resistência antimicrobiana (Luciani *et al*., 2023; Tesin *et al*., 2023).

De forma semelhante, a mastite bovina constitui uma das principais enfermidades da bovinocultura leiteira, afetando mais de 40% do rebanho bovino mundial e impactando diretamente a a qualidade do leite, o bem-estar animal, a segurança alimentar e a sustentabilidade da cadeia produtiva (Ghumman *et al.*, 2025). Sua etiologia multifatorial, envolvendo patógenos com elevada capacidade de persistência no tecido mamário através da resistência antimicrobiana e formação de biofilme, favorecem a manutenção de quadros clínicos e subclínicos e dificultam o controle da doença (Suthovski *et al*., 2024; Velasco Garcia *et al.*, 2025; Touaitia *et al*., 2025).

Paralelamente à busca por alternativas terapêuticas, a avaliação da segurança biológica desses compostos tem se tornado um aspecto central, uma vez que diversas substâncias investigadas como potenciais substitutos aos antimicrobianos convencionais têm demonstrado efeitos citotóxicos relevantes em células eucarióticas (Santa Catarina *et al.*, 2022). Nesse sentido, a investigação de moléculas que aliem eficácia antimicrobiana e antibiofilme a um perfil de segurança favorável torna-se essencial para o desenvolvimento de novas abordagens terapêuticas. Diante desse cenário, o presente estudo teve como objetivo avaliar toxicidade em células eucariontes e a atividade antimicrobiana e antibiofilme da nisina sob diferentes

contextos, com ênfase em sua ação frente a bactérias isoladas de casos de otite externa canina e de mastite bovina, contribuindo para o desenvolvimento de alternativas terapêuticas no enfrentamento da resistência antimicrobiana sob a perspectiva da Saúde Única (*One Health*).

2 REVISÃO DE LITERATURA

2.1 UMA SAÚDE, UM DESAFIO – A RESISTÊNCIA ANTIMICROBIANA NA INTERFACE ENTRE HUMANOS, ANIMAIS E O MEIO AMBIENTE:

A resistência antimicrobiana configura-se atualmente como uma das principais ameaças aos sistemas de saúde pública em escala global. Desde a descoberta dos primeiros fármacos e o usufruto de seus inúmeros benefícios, seu uso indiscriminado na medicina e medicina veterinária acelerou o ainda crescente fenômeno da resistência (Ferri *et al*., 2017). Em termos pecuniários, essa problemática resulta em perdas substanciais, decorrentes das elevadas taxas de morbidade e mortalidade, dos custos de internação hospitalar e dos impactos sociais associados (Bandyopadhyay; Samanta, 2020). No entanto, muitos dos riscos para a saúde ainda são intangíveis, especialmente no que se refere às projeções futuras e à subnotificação de casos, embora estudos indiquem que a resistência antimicrobiana represente um problema tão relevante quanto as doenças mais frequentemente relatadas em escala global (Westphal-Settele *et al*., 2018; Murray *et al*., 2022).

Do ponto de vista microbiológico, a resistência antimicrobiana pode ser classificada em três categorias principais: intrínseca, adaptativa e adquirida (Lee, 2019). A resistência intrínseca decorre das propriedades inerentes da bactéria, como a resistência a glicopeptídeos em bactérias Gram-negativas, atribuída à impermeabilidade de sua membrana externa. A resistência adaptativa, por sua vez, é induzida pela exposição a estresses externos, os quais impulsionam a expressão fenotípica de mecanismos de resistência, como a formação de biofilmes (Arzanlou; Chai; Venter, 2017). No contexto da medicina veterinária, o biofilme contribui significativamente para a tolerância aos antimicrobianos e para a persistência dos agentes etiológicos, incitando a cronicidade de casos de otite, dermatite e mastite (Chan *et al*., 2019; Pedersen *et al*., 2021; Stephen *et al.*, 2025).

Por fim, a resistência adquirida envolve a obtenção de mecanismos de resistência por bactérias previamente sensíveis, seja pela seleção de mutações ou pela aquisição de material genético exógeno (Christaki; Marcou; Tofarides, 2020). A elevada plasticidade genômica dos microrganismos favorece as interações entre a microbiota humana, animal e ambiental, resultando na disseminação de bactérias multirresistentes em diferentes nichos ecológicos (McEwen; Collignon, 2018; Aslam *et al*., 2021). Estudos demonstram que bactérias patogênicas incorporaram, ao longo do tempo, genes provenientes de bactérias ambientais por meio da transferência horizontal. Essa propagação entre sistemas pode ocorrer por inúmeras

vias, incluindo a agricultura, águas residuais, solos, produtos agropecuários, o contato direto entre humanos e animais e a cadeia alimentar (Graham *et al.*, 2019; Ahmad *et al*., 2021).

O solo é considerado um importante reservatório de genes de resistência a antimicrobianos, uma vez que a maioria dessas substâncias é derivada de microrganismos saprófitos intrinsecamente resistentes aos antibióticos que produzem. Além disso, a combinação de fatores como altas concentrações de antibióticos e/ou metais pesados em ambientes densamente colonizados por bactérias favorece a expressão e a propagação de mecanismos de resistência entre patógenos (Westphal-Settele *et al*., 2018). A água contaminada, por sua vez, pode atuar como importante vetor de disseminação da resistência antimicrobiana, ao carrear resíduos fecais e matéria orgânica potencialmente contaminados com bactérias resistentes, ampliando a circulação desses microrganismos entre diferentes compartimentos ambientais e populacionais (Prestinaci; Pezzotti; Pantosti, 2015; Bandyopadhyay; Samanta, 2020).

A interconexão entre humanos, animais e o meio ambiente cria condições favoráveis para a seleção e a disseminação da resistência antimicrobiana, sendo o uso indiscriminado de antimicrobianos um dos principais fatores que impulsionam esse processo, ao exercer pressão de seleção tanto sobre microrganismos patogênicos quanto comensais, que passam a atuar como reservatórios e disseminadores de genes de resistência (Ferri *et al.*, 2017). Além disso, a maioria das classes de antimicrobianos é utilizada de forma compartilhada no tratamento de infecções bacterianas em humanos e animais, bem como administrada em larga escala para fins de promoção de crescimento na suinocultura e avicultura (Prestinaci; Pezzotti; Pantosti, 2015; McEwen; Collignon, 2018).

O contato próximo entre animais de companhia e seres humanos favorece a transmissão interespecífica de bactérias e de seus genes de resistência, reforçando a interface epidemiológica proposta pelo conceito de Saúde Única (Grönthal et al., 2018; Marques et al., 2019; Martins et al., 2022). Nesse cenário, cães e gatos podem atuar como reservatórios de microrganismos multirresistentes, incluindo *Staphylococcus aureus* e *Staphylococcus pseudintermedius* resistentes à meticilina (MRSA e MRSP), *Enterococcus* resistentes à vancomicina (VRE) e enterobactérias produtoras de beta-lactamases de espectro estendido (ESBL) ou carbapenemases (Pomba *et al.*, 2017). Nessa mesma linha, estudos também vêm demonstrando níveis crescentes de resistência em isolados provenientes de animais de companhia, inclusive a antimicrobianos de uso restrito, como os carbapenêmicos, cuja aplicação deveria ser reservada a situações em que outras alternativas terapêuticas não se mostram eficazes (Silva *et al*., 2022; Monteiro *et al.,* 2025).

Entretanto, apesar dessa relevância, os animais de companhia permanecem sub-representados em programas estruturados de vigilância da resistência antimicrobiana, diferentemente do que ocorre na cadeia pecuária, em que a relação causal entre o uso de antimicrobianos e a seleção de resistência é mais consolidada (Guenin *et al*., 2022; Marco-Fuertes *et al*., 2022). Adicionalmente, estudos evidenciam o uso frequente de antimicrobianos criticamente importantes para a medicina, muitas vezes de forma empírica e sem respaldo em cultura e teste de sensibilidade (Saleem et al., 2019). Na prática clínica, esse panorama se reflete em aumento de falhas terapêuticas, recorrência de infecções e necessidade de utilização de terapias de resgate, com impacto direto no bem-estar animal e nos custos assistenciais, o que enquadra essa questão não como um fenômeno isolado, mas integrante de uma problemática sanitária de dimensão global (Weese *et al*., 2015; Ho *et al*., 2025).

Nesse contexto, organismos internacionais têm enfatizado a implementação de planos de ação globais fundamentados no princípio da Saúde Única como estratégia central para conter a progressão da resistência antimicrobiana. Considerando que a maioria dos antimicrobianos permanecerá disponível para uso tanto na medicina humana quanto na veterinária, impõe-se o desafio de otimizar a utilização desses medicamentos em escala global (WHO, 2017). Nesse sentido, torna-se necessária a adoção de abordagens integradas que preservem a eficácia dos antimicrobianos existentes, eliminem sua utilização inadequada e limitem o desenvolvimento e a disseminação de infecções (McEwen; Collignon, 2018; Aslam *et al.*, 2021). De forma complementar, substâncias com potencial antimicrobiano vêm sendo estudadas com o objetivo de ampliar o arsenal terapêutico disponível, incluindo bacteriocinas, bacteriófagos, probióticos e peptídeos antimicrobianos (Asaduzzaman; Sonomoto, 2009).

À luz do exposto, condutas fundamentadas no princípio da Saúde Única mostram-se essenciais para o enfrentamento efetivo da resistência antimicrobiana, implementadas de forma consistente por profissionais da medicina e medicina veterinária (Ferri *et al*., 2017). O uso de antimicrobianos dentro da medicina veterinária deve ser sustentável dentro do paradigma de Saúde Única e evoluir para usos mais prudentes e racionalmente fundamentados (Lees *et al.*, 2021). A resistência antimicrobiana transcende a esfera microbiológica, configurando-se como um fenômeno ecológico complexo, sustentado pela interdependência entre práticas clínicas, sistemas produtivos e compartimentos ambientais. Portanto, a corrida contra a resistência antimicrobiana constitui uma responsabilidade coletiva e demanda ações coordenadas em níveis local, nacional, regional e internacional, de modo a garantir a utilização sustentável dos antimicrobianos (Premanandh; Samara; Mazen, 2016).

2.2 OTITE EXTERNA CANINA – BASES PATOLÓGICAS E IMPLICAÇÕES PARA O TRATAMENTO MODERNO:

A otite externa é definida como a inflamação do canal auditivo externo, que abrange a orelha externa e os canais auditivos vertical e horizontal, até o nível do tímpano, e está entre as doenças mais prevalentes na população canina atendida na rotina, com prevalências relatadas de cerca de 15%, variando de acordo com a região geográfica, população estudada e critérios diagnósticos (Perry *et al.*, 2017; Pye, 2018; Kwon *et al.,* 2022). A ocorrência é maior em raças com orelhas pendulares, como Cocker Spaniel, Poodle e Golden Retriever, e pode apresentar variações sazonais, com picos em períodos de maior temperatura e umidade, além de maior prevalência em machos devido à influência dos andrógenos sobre a atividade glandular (Perry *et al.*, 2017; O'Neill *et al*., 2021; Li *et al.*, 2023).

Além de sua elevada prevalência, a otite externa impacta significativamente o bem-estar animal, a qualidade de vida dos tutores e os custos terapêuticos. A dor, o prurido e o desconforto crônico comprometem a qualidade de vida do cão acometido, enquanto tratamentos prolongados e recorrentes geram ônus financeiro e emocional aos tutores (Li *et al*., 2023). Clinicamente, a alta frequência de isolamentos bacterianos, associada à multirresistência, à presença de isolados resistentes a medicamentos de importância e a taxas de recorrência de até 24%, mesmo após tratamento inicial adequado, evidencia o caráter crônico da doença e amplia sua relevância no contexto da resistência antimicrobiana e da saúde única (Perry *et al.*, 2017; Tesin *et al.*, 2023).

Os fatores envolvidos na patogenia da otite externa podem ser classificados em predisponentes, primários e perpetuantes (Brame; Cain, 2021). Os fatores predisponentes correspondem a características ambientais, anatômicas ou fisiológicas que, embora não causem inflamação por si só, aumentam o risco de desenvolvimento da doença ao alterarem o microambiente do canal auditivo, tornando-o mais favorável à proliferação de microrganismos oportunistas e à instalação do processo inflamatório (Saengchoowong *et al.*, 2023; Núnez *et al*., 2025). Entre esses fatores destacam-se a conformação do canal auditivo, o excesso de pelos e a hipersecreção de cerúmen - frequentemente associados à raça, bem como alterações da microbiota residente decorrentes de doenças prévias ou terapias anteriores, higienização excessiva com ou sem arrancamento de pelos, variações de temperatura e umidade do conduto auditivo e presença de massas (Bajwa, 2019).

Os fatores primários abrangem afecções que acometem diretamente o canal auditivo e são responsáveis pela instalação inicial do processo inflamatório, como infecções bacterianas e

fúngicas, parasitoses, doenças endócrinas, como hipotireoidismo e hiperadrenocorticismo, doenças imunomediadas, como dermatite atópica e hipersensibilidade alimentar, neoplasias e corpos estranhos (Paterson; Matyskiewicz, 2018; Brame; Cain, 2021; O'Neill *et al.*, 2021; Nuttall, 2023). Quando de origem infecciosa, a otite apresenta etiologia frequentemente polimicrobiana, envolvendo principalmente bactérias oportunistas que se beneficiam do microambiente alterado, como *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis* e *Streptococcus* spp., além de leveduras como *Malassezia pachydermatis* (Bourély *et al*., 2019; Martins *et al*., 2022). A interação dinâmica entre fatores predisponentes e primários promove alterações estruturais e funcionais do epitélio do canal auditivo, resultando em edema, aumento da produção de exsudato e ruptura da barreira cutânea, o que favorece a colonização microbiana e cria as condições ideais para a atuação dos fatores perpetuantes (Bajwa, 2019).

Os fatores perpetuantes correspondem a alterações que mantêm e agravam o processo inflamatório, mesmo após a correção da causa primária, contribuindo para a cronificação da otite externa e evolução para otite média e/ou interna, que aumentam a complexidade e riscos do tratamento (Gotthelf, 2004; Souza *et al.*, 2023). A formação de biofilmes e o desenvolvimento de resistência antimicrobiana, especialmente em resposta à prescrição empírica de antimicrobianos, prática comum na rotina clínica, exercem papel central na manutenção do quadro, dificultando a erradicação da infecção (Bajwa, 2019; Kwon *et al*., 2022; Martins *et al*., 2022; Hobi *et al.,* 2024; Savaliya *et al.,* 2025; Stephen *et al.,* 2025). A inflamação crônica promove espessamento epitelial, hiperplasia glandular e redução do lúmen do conduto auditivo, criando um microambiente úmido, pobremente oxigenado e rico em detritos orgânicos, tornando o contexto clínico ainda mais favorável à persistência bacteriana (Bajwa *et al*., 2019; Nuttall, 2023).

O biofilme é uma matriz polimérica extracelular organizada, composta por polissacarídeos, proteínas e DNA extracelular, que envolve as células microbianas e atua como barreira física e funcional contra agentes antimicrobianos e a resposta imune do hospedeiro, sendo reconhecido como um dos principais mecanismos envolvidos na cronificação e refratariedade ao tratamento (Robinson *et al*., 2019; Rather; Gupta; Mandal, 2021). Estudos utilizando microscopia eletrônica de varredura já confirmaram a presença de biofilme *in vivo* em casos de otite externa, embora sua detecção clínica e citológica seja limitada, indicando que o biofilme provavelmente é subdiagnosticado na prática veterinária (Luciani *et al*., 2023). Após instaurado, o biofilme favorece a exposição prolongada a concentrações subinibitórias de antimicrobianos, incitando pressão seletiva e amplificação de mecanismos de resistência (Schilcher; Horswill, 2020).

A resistência antimicrobiana em patógenos associados à otite externa canina representa um desafio crescente na prática clínica, especialmente diante da elevada prevalência da doença e de seu caráter recorrente. *S. pseudintermedius*, *S. aureus* e *Proteus mirabilis* figuram entre os principais agentes isolados com perfil multirresistente, com baixa resposta a β-lactâmicos, cefalosporinas, aminoglicosídeos e fluoroquinolonas, além de *Pseudomonas aeruginosa*, com resistência intrínseca aos dois primeiros e elevada prevalência de isolados *pandrug resistant* (PDR) (Shen; Zhu; Wang, 2008; Bourély *et al*., 2019; Saraiva *et al*., 2025; Wang *et al.*, 2025). A ocorrência de infecções mistas e a progressão temporal dos padrões de resistência evidenciam as limitações da terapia empírica e reforçam a necessidade de abordagem baseada em cultura e teste de suscetibilidade, além de estratégias que considerem o impacto da resistência no contexto da Saúde Única (Garcias *et al*., 2025).

O diagnóstico laboratorial da otite apresenta limitações inerentes às técnicas disponíveis. A avaliação citológica do exsudato auricular é útil para orientar e monitorar a evolução da otite externa (Choi *et al*., 2018). A cultura microbiológica continua sendo amplamente utilizada por permitir a identificação de patógenos e a avaliação da suscetibilidade antimicrobiana, embora não detecte microrganismos não cultiváveis. Técnicas moleculares ampliam a identificação da diversidade bacteriana, mas ainda não são aplicáveis rotineiramente na prática clínica. Dessa forma, a interpretação diagnóstica deve considerar a complementaridade entre os métodos e o contexto clínico do paciente (Leonard *et al*., 2022).

O tratamento da otite externa canina requer a identificação e correção dos fatores primários, predisponentes e perpetuantes. A terapia tópica com antimicrobianos, antifúngicos e glicocorticoides é geralmente eficaz a curto prazo, mas ciclos repetidos de inflamação podem favorecer alterações estruturais irreversíveis e progressão para cronicidade (Bajwa, 2019; Nuttall, 2019). Além disso, a recorrência mesmo após tratamento adequado sugere que a erradicação bacteriana frequentemente não é completa, especialmente em casos associados à formação de biofilme e a alterações estruturais permanentes do conduto auditivo (Bjarnsholt, 2013). O manejo deve contemplar fases de indução e manutenção, com limpeza auricular, controle da infecção e modulação da inflamação. Embora a limpeza física do conduto possa reduzir a carga bacteriana, o sucesso terapêutico depende principalmente do controle da causa primária (Corb *et al.*, 2024).

Diante da complexidade multifatorial desse tema, torna-se evidente a limitação das abordagens terapêuticas convencionais baseadas exclusivamente em antimicrobianos clássicos (Bajwa, 2019). Esse cenário tem impulsionado a investigação de estratégias alternativas, como peptídeos antimicrobianos, óleos essenciais, extratos vegetais, sistemas de liberação controlada,

agentes com atividade antibiofilme e formulações capazes de potencializar a eficácia de fármacos existentes (De Martini *et al.,* 2021; Bannach *et al.*, 2025).

2.3 MASTITE BOVINA – MECANISMOS DE INFECÇÃO, PERSISTÊNCIA BACTERIANA E LIMITAÇÕES DO TRATAMENTO:

A mastite é definida como a inflamação do tecido mamário decorrente de danos físicos, irritação química ou, predominantemente, ação de microrganismos invasores (Ashraf; Imran, 2020; Velasco Garcia *et al*., 2025). Dentro da bovinocultura leiteira, é considerada a doença mais frequente e economicamente relevante, com impacto negativo direto na produção, composição e qualidade do leite (Gomes; Henriques, 2016), além de gastos indiretos associados a cuidados veterinários, administração de medicamentos e descarte de leite contendo resíduos de antimicrobianos (Zhylkaidar *et al*., 2021; Caneschi *et al*., 2023).

A etiologia da mastite é diversa, envolvendo microrganismos de origem contagiosa e ambiental. Entre os agentes contagiosos, destacam-se *Staphylococcus aureus*, *Streptococcus agalactiae*, *Staphylococcus* não-aureus e *Corynebacterium* spp., transmitidos principalmente durante a ordenha e associados à capacidade de colonização persistente da glândula mamária (Zaatout; Ayachi; Kecha, 2020). Os agentes ambientais incluem coliformes, como *Escherichia coli* e *Klebsiella* spp., *Streptococcus uberis*, *Trueperella pyogenes* e *Pseudomonas* spp., relacionados à contaminação do úbere pelo ambiente (Svennesen *et al*., 2023). Microrganismos como *Mycoplasma* spp., *Prototheca* spp., *Nocardia* spp. e leveduras do gênero *Candida* spp. são frequentemente associados a infecções refratárias e à cronicidade (Piaia *et al*., 2025).

A glândula mamária possui um sistema imunológico especializado cuja função é prevenir a invasão bacteriana, eliminar patógenos e restaurar a integridade tecidual (Mavangira, 2025). Essa defesa baseia-se em uma rede integrada de mecanismos físicos, celulares e solúveis, geridos predominantemente pela imunidade inata, ativada rapidamente após a exposição aos microrganismos (Stanek; Żółkiewski; Januś, 2024). A barreira da extremidade do teto, o recrutamento de células inflamatórias e a liberação de mediadores imunológicos são essenciais para conter a infecção nas fases iniciais. Contudo, essa resposta é pouco específica e não gera memória imunológica, exigindo equilíbrio entre ativação e resolução para evitar danos teciduais (Sordillo, 2018).

A patogênese da mastite bovina tem início com a penetração do microrganismo pelo canal do teto, migrando pela cisterna até atingir os alvéolos mamários, onde utiliza fatores de

virulência específicos para adesão e colonização epiteliais (Petersson-Wolfe; Mullarky; Jones, 2010; Tong *et al*., 2025). No caso de *S. aureus*, as proteínas do grupo das moléculas da matriz adesiva que reconhecem o componente de superfície (MSCRAMMs) desempenham papel central na fixação aos componentes da matriz extracelular, enquanto bactérias Gram-negativas utilizam fímbrias e proteínas de membrana externa para estabelecer a infecção (Foster *et al.*, 2014).

As células epiteliais mamárias bovinas (bMECs) atuam como alvos primários da infecção e como sensores iniciais, reconhecendo padrões moleculares associados a patógenos (PAMPs) por meio de receptores do tipo Toll (TLRs), como o peptideoglicano das bactérias Gram-positivas pelo TLR2 e o lipopolissacarídeo (LPS) das bactérias Gram-negativas pelo TLR4 (Oviedo-Boyso *et al*., 2007; Tong *et al*., 2025). O reconhecimento dos patógenos pelas bMECs desencadeia a ativação de vias de sinalização intracelular, como o fator nuclear kappa B (NF-κB) e Mitogen-Activated Protein Kinases (MAPK), culminando na liberação de citocinas pró-inflamatórias, incluindo IL-1β, IL-6, IL-8 e TNF-α, que promovem o recrutamento de células do sistema imune para o sítio da infecção (Gilbert *et al*., 2013).

Para sobreviver e proliferar no ambiente mamário, os microrganismos lançam mão de diferentes estratégias de evasão e agressão tecidual - bactérias produzem exotoxinas e enzimas, como hemolisinas e o fator CAMP, capazes de causar lise celular e danos ao tecido glandular, além de utilizar sistemas eficientes de aquisição de ferro, como os sideróforos, para se multiplicarem em um ambiente pobre em ferro biodisponível, como o leite (Abril *et al*., 2020; Aslam *et al.*, 2021; Tong *et al*., 2025). Adicionalmente, a formação de biofilme, especialmente associada a *S. aureus*, confere proteção contra a ação do sistema imune e dos antimicrobianos, favorecendo a manutenção do foco infeccioso, a recorrência dos quadros clínicos e a evolução para a cronicidade (Sharifi; Mahmoudi; Sobhani, 2024).

Na glândula mamária, os biofilmes podem se estabelecer tanto na superfície do epitélio alveolar quanto nos ductos lactíferos, formando microambientes protegidos que dificultam a penetração de antibióticos e a ação de células de defesa (Stewart; Costerton, 2001; Bjarnsholt, 2013). Essa organização estrutural, associada ao metabolismo reduzido das bactérias em biofilme e à expressão diferencial de genes de resistência, compromete a eficácia dos tratamentos convencionais (Demontier *et al.*, 2025). Como consequência, infecções associadas a biofilme apresentam persistência intramamária e baixa taxa de cura bacteriológica, incitando a remoção voluntária do rebanho (Lewis, 2001; Scherr *et al*., 2014; Bellato *et al*., 2023).

A interação entre esses fatores resulta em uma manifestação clínica ou subclínica, de acordo com a presença ou ausência de sinais clínicos evidentes. A forma clínica é caracterizada

por alterações inflamatórias visíveis no leite e no tecido mamário, incluindo grumos, aquosidade, presença de exsudato purulento ou sangue, além de sinais locais como edema, calor, dor e alterações comportamentais (Peters; Silveira; Fischer, 2015; Petersson-Wolfe; Leslie; Swartz, 2018). Em quadros mais graves, podem ocorrer sinais sistêmicos, como febre, apatia, anorexia e queda abrupta da produção. Em contraste, a mastite subclínica não apresenta sinais clínicos aparentes, sendo identificada por testes que mensuram a inflamação da glândula mamária e a composição do leite (Swami; Patil; Gadekar, 2017; Kour *et al*., 2023).

Além do efeito negativo no bem-estar animal, a mastite impacta diretamente o valor nutricional e as propriedades tecnológicas do leite, incitando níveis elevados de sódio, cloreto, ácidos graxos livres e frações proteicas não caseínicas, bem como menores teores de caseína, lactose, potássio e cálcio (Zalewska *et al.*, 2025). Evidências também apontam para prejuízo ao rendimento e qualidade de derivados lácteos, com prolongamento do tempo de coagulação e pior qualidade da coalhada, especialmente nos casos de mastite associada a patógenos contagiosos como *S. aureus* e *S. agalactiae* (Bobbo *et al*., 2017).

O diagnóstico da mastite bovina baseia-se na avaliação clínica e em métodos de mensuração da resposta inflamatória da glândula mamária, destacando-se a contagem de células somáticas (CCS) e o *California Mastitis Test* (CMT) (Ashraf; Imran, 2018; El-Sayed; Kamel. 2021). A cultura microbiológica permanece como método de referência para o diagnóstico da infecção intramamária (Adkins; Middleton, 2018), entretanto, sistemas de cultura realizados na própria propriedade (*on-farm culture*) têm ganhado relevância por possibilitarem a identificação preliminar dos patógenos e a tomada de decisão terapêutica de forma mais rápida, direcionada e racional (Zadoks *et al*., 2023). Paralelamente, técnicas moleculares, como a reação em cadeia da polimerase (PCR) e o sequenciamento do gene 16S rRNA, vêm sendo cada vez mais empregadas devido à sua elevada sensibilidade e rapidez na detecção bacteriana (Usui *et al*., 2023).

O tratamento da mastite bovina baseia-se predominantemente no uso de antimicrobianos, principalmente penicilinas e cefalosporinas, tanto durante a lactação quanto na terapia de vaca seca. Contudo, o uso frequente e, muitas vezes, empírico desses fármacos tem sido questionado, uma vez que parte das infecções apresenta resolução espontânea ou envolve patógenos pouco responsivos (Piaia *et al.*, 2025). A terapia intramamária, apesar de permitir altas concentrações locais, apresenta limitações importantes, como distribuição desigual no tecido mamário e baixa penetração em áreas profundas da glândula, especialmente na presença de biofilme (Pyörälä, 2009). Adicionalmente, fatores como o pH do leite e as

propriedades farmacocinéticas dos fármacos podem comprometer a manutenção de concentrações terapêuticas eficazes (Louhi *et al*., 1992).

Somando-se ao biofilme, a resistência antimicrobiana tem se consolidado como um dos principais entraves no controle da mastite bovina. Estudos demonstram ampla variabilidade nas taxas de resistência de *S. uberis* a penicilina, eritromicina e tetraciclina (Miotti *et al.*, 2023). De forma semelhante, *S. aureus* apresenta elevadas taxas de resistência, particularmente à penicilina, com tendência crescente ao longo das últimas décadas, sobretudo em regiões da África, Ásia e América Latina (Molineri *et al*., 2021). A exposição recorrente a doses subinibitórias de antimicrobianos, especialmente em patógenos formadores de biofilme, contribui para a seleção e manutenção de isolados resistentes (Efimochkina *et al.,* 2018).

Diante desses desafios, outros regimes terapêuticos têm sido avaliados quanto à sua eficácia no tratamento da mastite, como a aplicação de bacteriocinas, bacteriófagos, peptídeos antimicrobianos, probióticos, fitoterápicos, nanopartículas, entre outros (Algharib *et al.*, 2020; Sharun *et al.*, 2021; Raheel; Mohammed; Mohamed, 2023). Nesse contexto, a nisina tem se destacado por sua atividade contra células planctônicas e na redução da viabilidade de biofilmes (Ceotto-Vigoder *et al.*, 2016). Evidências recentes também indicam efeito anti-inflamatório pela inibição de vias MAPK, redução de citocinas pró-inflamatórias e fortalecimento da barreira hemato-láctea (Huang *et al*., 2022). Além disso, estratégias de bioengenharia têm permitido o desenvolvimento de derivados com maior especificidade contra patógenos associados à mastite, sem comprometer bactérias lácticas benéficas (Field *et al*., 2021).

2.4 NISINA ALÉM DOS ALIMENTOS – AVANÇOS E PERSPECTIVAS TERAPÊUTICAS:

Hoje, frente ao crescente processo de resistência antimicrobiana, acredita-se que os lantibióticos, grupo de peptídeos antibióticos contendo lantionina, apresentem potencial considerável como alternativas terapêuticas (Musiejuk; Kafarski, 2023). A nisina é uma bacteriocina lantibiótica tipicamente produzida por *Lactococcus lactis*, relatada pela primeira vez em 1928 como uma substância inibidora hipotética, sendo reconhecida oficialmente em 1947, após seu isolamento no leite (Rogers; Whittier, 1928; Mattick; Hirsch; Berridge, 1947). Em 1988, foi aprovada como conservante alimentar pela *Food and Drug Administration* (FDA) por sua atividade antimicrobiana consolidada contra uma variedade de microrganismos patogênicos e deteriorantes transmitidos por alimentos (Oliveira *et al*., 2024).

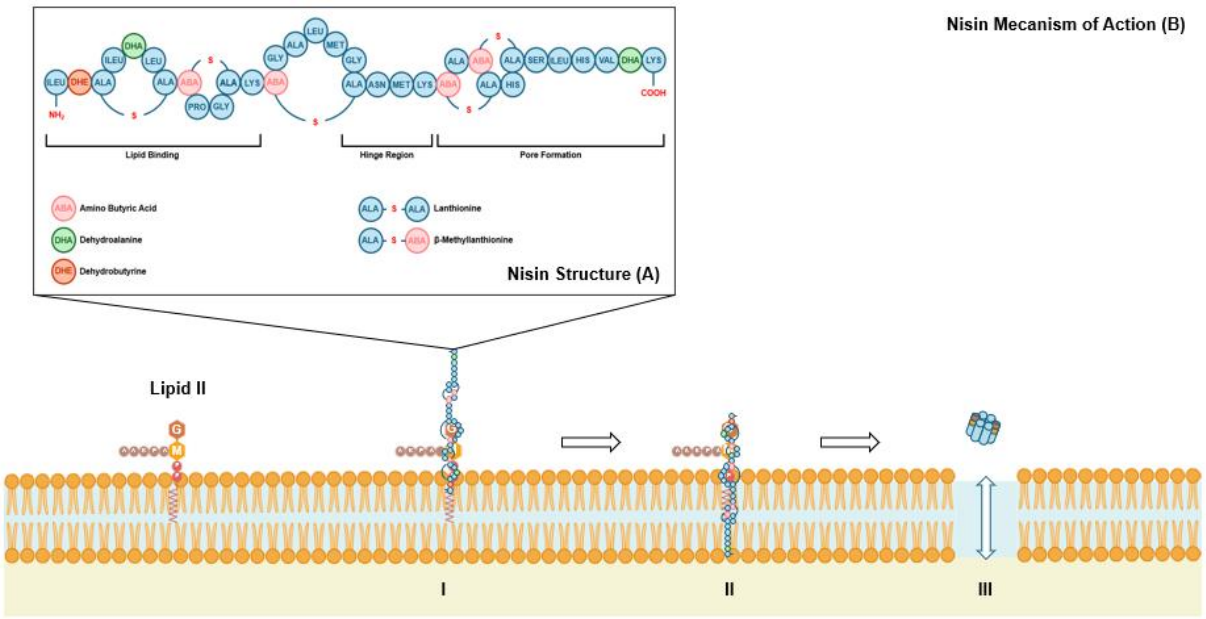
Estruturalmente, a nisina é um peptídeo catiônico relativamente pequeno, composto por 34 aminoácidos e com massa molecular aproximada de 3 a 3,5 kDa. Caracteriza-se pela presença de cinco pontes de lantionina, responsáveis por conferir à molécula uma conformação tridimensional rígida e altamente estável, fundamental para sua atividade antibacteriana (Gross; Morell, 1971). Sua estrutura também inclui aminoácidos incomuns, como desidroalanina, desidrobutirina e β-metil-lantionina, formados a partir de modificações pós-traducionais de um polipeptídeo precursor sintetizado ribossomicamente, que garantem a estabilidade estrutural e a interação específica com alvos bacterianos (Musiejuk; Kafarski, 2023). Esse arranjo pode ser observado na figura 1A.

Além disso, a nisina apresenta dois domínios estruturais funcionalmente distintos, que possibilitam seu mecanismo de ação dupla. O domínio N-terminal exibe alta afinidade pelo grupo pirofosfato do lipídio II, com o qual interage formando uma estrutura altamente estável, sustentada por ligações de hidrogênio intermoleculares. Em contraste, o domínio C-terminal projeta-se em direção à bicamada fosfolipídica, favorecendo a inserção do peptídeo na membrana citoplasmática (Hart *et al., 2015*). As regiões N- e C-terminais são conectadas por uma curta e flexível região de dobradiça (*hinge region*), que permite a translocação do domínio C-terminal através da membrana celular (Musiejuk; Kafarski, 2023).

Através dessa estrutura, a ação antimicrobiana da nisina se dá primariamente por essa interação altamente direcionada ao lipídio II, um precursor essencial na biossíntese de peptideoglicano da parede celular bacteriana (Sahl; Bierbaum, 2008; Zaschke-Kriesche *et al*., 2019). A presença desse alvo molecular modifica substancialmente a energética e a arquitetura do processo de formação de poros, reduzindo de forma significativa o potencial elétrico necessário para a inserção do peptídeo na bicamada lipídica. Os poros formados são estruturas estáveis e bem definidas, com diâmetros estimados entre 2 e 2,5 nm, resultantes da associação de múltiplos complexos estequiométricos de nisina e lipídio II (Wiedemann et al., 2004).

As consequências fisiológicas dessa formação são rápidas e pronunciadas, culminando na morte celular abrupta em decorrência da perda da integridade da membrana citoplasmática, efluxo de compostos intracelulares e despolarização completa da membrana. Esse colapso eletroquímico e osmótico resulta na interrupção dos processos biossintéticos fundamentais, tornando a célula bacteriana incapaz de manter sua viabilidade (Ruhr; Sahl; Hans, 1985; Sahl; Kordel; Benz, 1987). Esses processos podem ser visualizados na figura 1B.

Figura 1 – Estrutura química (A) e mecanismo de ação da nisina contra bactérias (B).



(I) reconhecimento e ligação específica do domínio N-terminal da nisina ao lipídio II na membrana citoplasmática bacteriana, resultando no sequestro desse precursor essencial da biossíntese do peptidoglicano. (II) Reorientação conformacional da molécula, mediada pela hinge region, permitindo a inserção do domínio C-terminal na bicamada lipídica. (III) Formação de poros transmembrana estáveis a partir do complexo nisina-lipídio II. Fonte: Criado com BioRender.com (2026).

Devido ao seu mecanismo de ação intrinsecamente relacionado à parede celular, a nisina é ativa contra uma ampla variedade de bactérias gram-positivas, como *Lactococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp., *Micrococcus* sp. e *Listeria* sp., bem como formas vegetativas e esporos em crescimento de *Bacillus* sp. e *Clostridium* sp. (McAuliffe; Ross; Hill, 2001; Punyauppa-Path; Phumkhachorn; Rattanachaikunsopon; 2015; Aveyard *et al*., 2017). Por outro lado, a presença de membranas externas ricas em lipopolissacarídeos (LPS) em bactérias-gram negativas limita sua penetração (Vukomanović *et al.*, 2017), assim como a complexa organização da parede celular de bactérias ricas em ácidos micólicos, cuja elevada fração lipídica atua como barreira física à difusão do peptídeo e restringe o acesso ao seu alvo molecular (Carroll *et al.*, 2010; Ali *et al.*, 2019).

Além do espectro antimicrobiano restrito, a aplicação da nisina, particularmente por via sistêmica, apresenta limitações adicionais, como sua meia-vida circulante curta decorrente da rápida filtração renal, suscetibilidade à degradação enzimática, captação pelo sistema reticuloendotelial e possível acúmulo em órgãos e tecidos não-alvo (Torchilin; Lukyanov, 2003; Asaduzzaman; Sonomoto, 2009). Em aplicações tópicas, especialmente no contexto do manejo de feridas, sua eficácia pode ser comprometida tanto por fatores ambientais - incluindo

hidrólise, oxidação e fotólise - quanto por condições intrínsecas ao leito da ferida, como pH alcalino e elevada atividade proteolítica (Thapa; Diep; Tønnesen, 2020).

Para além de sua atividade antimicrobiana já consolidada, estudos têm demonstrado sua ação moduladora multifatorial no sistema imune. Essas ações incluem a ativação de diversas vias de sinalização celular, como NF-κB, ERK/MAPK, PKC e PKA, estímulo à produção de interleucinas, como IL-6 e IL-8, MCP-1, Gro-α e quimiocinas, em células em repouso, (Kindrachuk *et al.*, 2013). Em modelos de estimulação inflamatória, a nisina promove efeito anti-inflamatório ao reduzir citocinas pró-inflamatórias, como TNF-α, IL-1β, IL-6 e IL-12p70 (Uehara; Maekawa; Nakagawa, 2025), e aumentar citocinas reguladoras, como IL-4, IL-10 e IFN-γ (Jia *et al.*, 2019; Małaczewska *et al.,* 2019; Mouritzen *et al.,* 2019).

Na imunidade inata, a nisina pode aumentar os níveis de monócitos e neutrófilos periféricos e a formação de armadilhas extracelulares de neutrófilos (NETs). Já na imunidade adaptativa, promove o aumento transitório de linfócitos T CD4+ e CD8+, com redução concomitante de linfócitos B, a indução de efeitos antiproliferativos em linfócitos ativados por mitógenos e possivelmente a evasão contra a apoptose, prevenindo a linfopenia (Pablo *et al*., 1999; Brand; Smith; Dicks, 2013; Begde *et al*., 2011; Małaczewska *et al.,* 2019). Além disso, apresenta ação antioxidante ao elevar a atividade das enzimas superóxido-dismutase (SOD) e catalase (CAT) quando combinada a antimicrobianos, neutralizando as espécies reativas de oxigênio, protegendo contra dano oxidativo celular e atenuando as vias inflamatórias ligadas ao estresse oxidativo (Villamil; Figueiras; Novoa, 2003; Singh; Preet; Rishi, 2014).

Além da resistência intrínseca de bactérias Gram-negativas, diversos mecanismos de resistência à nisina têm sido descritos em bactérias Gram-positivas, envolvendo principalmente alterações estruturais e regulatórias da célula bacteriana, bem como a expressão de proteínas específicas de defesa (Draper *et al.*, 2015; Khosa; Lagedroste; Smits, 2016). Um desses mecanismos é a D-alanilação dos ácidos teicoicos associada ao espessamento da parede celular, que reduzem a interação eletrostática e dificultam o acesso da nisina ao seu alvo (Peschel *et al*., 1999). Modificações na estrutura ou na disponibilidade do lipídio II na membrana citoplasmática também podem diminuir a afinidade de ligação da nisina, comprometendo tanto a inibição da síntese de peptidoglicano quanto a formação de poros na membrana (Kramer *et al.,* 2006).

A ação de proteases específicas pode resultar na clivagem e inativação da nisina, diminuindo sua estabilidade e atividade antimicrobiana, especialmente em ambientes com exposição prolongada ao peptídeo (Pan *et al*., 2020). Ainda, os sistemas de efluxo compostos pela proteína NisFeg (sistema ABC de transporte), atuam promovendo a remoção ativa da

nisina do meio intracelular ou pericelular, reduzindo sua concentração efetiva próxima à membrana citoplasmática (Carniello *et al*., 2018).

Algumas bactérias também apresentam o Sistema de resitência à nisina (NRS), um mecanismo de resistência ativado em resposta à exposição à nisina mesmo em concentrações subletais (Porta *et al*., 2019). A ativação do sistema culmina na expressão da proteína efetora de resistência à nisina (NrsA), que atenua a interação do peptídeo com a membrana celular e com o lipídio II. Esse mecanismo não confere imunidade completa nem envolve a degradação direta do peptídeo, mas favorece a sobrevivência e adaptação transitória sob pressão seletiva contínua, contribuindo para a persistência celular em ambientes contendo nisina em níveis subinibitórios (Sun *et al.*, 2009).

Peptídeos pentacíclicos como a nisina têm sido utilizados com sucesso como conservantes na indústria alimentícia, recebendo atenção mais recentemente quanto à exploração do seu potencial clínico, tendo em vista à crescente resistência às formulações antimicrobianas disponíveis no mercado (Yuan *et al.,* 2024). Estudos prévios demonstraram eficácia no tratamento de mastite bovina, cáries dentárias, câncer e infecções de pele – inclusive contra isolados de prioridade máxima, como *Staphylococcus aureus* resistente à meticilina (MRSA) - o que implica potencial terapêutico tanto para a medicina quanto para a medicina veterinária (Kamarajan *et al.,* 2015; Shin *et al.,* 2016; Guan *et al.,* 2017; Jensen *et al.*, 2020; Wang *et al*., 2023).

Evidências recentes demonstram que a associação da nisina a outros compostos antimicrobianos, naturais ou sintéticos, pode não apenas intensificar sua atividade, mas também promover a ampliação de seu espectro de ação, alcançando microrganismos tradicionalmente menos suscetíveis (Wang *et al*., 2023). Combinações com óleos essenciais, peptídeos antimicrobianos e antibióticos convencionais têm apresentado efeitos sinérgicos relevantes contra patógenos como *P. aeruginosa* e *Salmonella enterica*, incluindo a inibição de biofilmes, a redução da expressão de fatores de virulência e a restauração da sensibilidade antimicrobiana (He *et al*., 2025; Karaca *et al.,* 2025; Preet *et al*., 2025).

Paralelamente, avanços em estratégias de formulação e em sistemas baseados em nanotecnologia têm sido explorados para superar as limitações físico-químicas e potencializar a eficácia da molécula frente a microrganismos de maior complexidade estrutural. A nanoencapsulação e a coencapsulação com outros compostos bioativos têm demonstrado capacidade de aumentar a estabilidade, a biodisponibilidade e a penetração do peptídeo, configurando estratégias promissoras para a ampliação do uso terapêutico da nisina além do seu espectro clássico (Flynn; Ryan; Hudson, 2022; Quichaba et al., 2023).

3 PRIMEIRO EXPERIMENTO

EVALUATION OF TOXICITY, ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY OF NISIN AGAINST BACTERIA ISOLATED FROM CANINE OTITIS EXTERNA

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RESUMO

A otite externa canina está entre as doenças mais prevalentes e desafiadoras na prática clínica, exercendo impacto significativo no bem-estar animal e na saúde pública. A nisina, um lantibiótico utilizado na indústria alimentícia, já demonstrou atividade antimicrobiana e antibiofilme no passado, mas sua aplicação no contexto da otite externa canina permanece inexplorada. O objetivo do presente estudo foi avaliar a atividade antimicrobiana, antibiofilme e da nisina contra bactérias isoladas de otite externa canina, bem como sua toxicidade em células eucariontes, visando sua aplicação futura como alternativa terapêutica. 34 isolados bacterianos foram obtidos a partir de cães diagnosticados com otite externa, incluindo

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Corynebacterium spp. e *Staphylococcus* spp. coagulase-negativa, os quais foram previamente triados quanto ao seu perfil de suscetibilidade antimicrobiana. Para avaliação da eficácia da nisina, foram avaliados a concentração inibitória mínima (CIM), a concentração bactericida mínima (CBM), a curva de crescimento e os ensaios de cinética de morte bacteriana. Ensaios de formação, inibição e erradicação de biofilme também foram realizados conforme descrito pela literatura. A avaliação de toxicidade em células eucariontes foi estimada pelo percentual de hemólise. A nisina apresentou atividade antimicrobiana variável, com inibição de 82% dos isolados de *Corynebacterium* spp. e 25% dos *Staphylococcus* spp. coagulase-negativos. A atividade bactericida foi limitada, sendo observada apenas em dois isolados de *Corynebacterium* spp. e ausente em *Staphylococcus* spp. Houve redução significativa do crescimento bacteriano ao longo do tempo, com recuperação parcial posterior. A nisina reduziu a formação de biofilmes, especialmente em isolados moderados e fortes, mas não promoveu sua erradicação. Além disso, apresentou baixa toxicidade, com índice de hemólise de 0,20%. Apesar desses resultados promissores, são necessários mais estudos acerca da nisina, para definir sua aplicabilidade terapêutica na otite externa canina.

Palavras-chave: resistência antimicrobiana; peptídeos; sanidade animal; dermatologia veterinária; inflamação; conduto auditivo; cão.

ABSTRACT

Canine otitis externa is among the most prevalent and challenging diseases in clinical practice, significantly impacting animal welfare and public health. Nisin, a lantibiotic used in the food industry, has demonstrated antimicrobial and antibiofilm activity in the past, but its application in the context of canine otitis externa remains unexplored. The aim of this study was to evaluate the antimicrobial and antibiofilm activity of nisin against bacteria isolated from canine otitis externa, as well as its toxicity in eukaryotic cells, aiming at its future application as a therapeutic alternative. 34 bacterial isolates were obtained from dogs diagnosed with otitis externa, including Corynebacterium spp. and coagulase-negative Staphylococcus spp., which were previously screened for their antimicrobial susceptibility profile. To evaluate the efficacy of nisin, the minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), the growth curve, and bacterial death kinetics assays were assessed. Biofilm formation, inhibition, and eradication assays were also performed as described in the literature. Toxicity

in eukaryotic cells was estimated by the percentage of hemolysis. Nisin showed variable antimicrobial activity, inhibiting 82% of *Corynebacterium* spp. isolates and 25% of coagulase-negative *Staphylococcus* spp. isolates. Bactericidal activity was limited, observed only in two *Corynebacterium* spp. isolates and absent in *Staphylococcus* spp. There was a significant reduction in bacterial growth over time, with subsequent partial recovery. Nisin reduced biofilm formation, especially in moderate and strong isolates, but did not promote its eradication. Furthermore, it showed low toxicity, with a hemolysis index of 0.20%. Despite these promising results, further studies on nisin are needed to define its therapeutic applicability in canine otitis externa.

Keywords: antimicrobial resistance; peptides; animal sanity; veterinary dermatology; inflammation; ear canal; dog.

3.1 INTRODUCTION

Canine otitis externa is among the most prevalent and challenging diseases in small animal practice, exerting a significant impact on animal welfare and presenting relevance to public health (Li *et al*., 2023). Its etiology is frequently polymicrobial and closely associated with antimicrobial resistance and biofilm-forming capacity, factors that difficult treatment and contribute to disease recurrence and chronicity, witch is observed in approximately 24% of cases (Perry *et al*., 2017; Tesin *et al*., 2023). *Corynebacterium* species and coagulase-negative *Staphylococcus* spp., although usually considered commensals bacteria, have emerging as opportunistic pathogens in canine otitis due to these virulence and resistance factors (Lee *et al*., 2019; Vinhal *et al*., 2024).

Topical treatment is considered successful in the short term, but repeated cycles of inflammation and infection lead to chronic inflammatory changes, witch can, eventually, become irreversible and require a total ear canal ablation (Nuttall, 2023). As empirical antimicrobial therapy is commonly employed in these cases, the emergence of antimicrobial resistance represents an additional complicating factor in clinical management and antimicrobial resistance dissemination, due to proximal contact between humans and animals (Martins *et al*., 2022).

In this context, alternative therapies have been investigated for their pharmacological potential, with antimicrobial peptides standing out as promising strategies in view of the

limitations of conventional treatments (De martini *et al.*, 2021; Bannach *et al*., 2025). Nisin, a bacteriocin widely applied in the food industry since the 1980s, is well established in the literature for its antimicrobial and antibiofilm activity (Oliveira *et al*., 2024), however, no studies have investigated the activity of nisin against bacteria isolated from canine otitis externa yet. Therefore, the aim of the present study was to evaluate the antimicrobial and antibiofilm activity of nisin against bacteria isolated from canine otitis externa, as well as its toxicity in eukaryotic cells, in order to contribute to the development of more effective therapeutic strategies for the management of this condition.

3.2 MATERIAL AND METHODS

2.1 Sampling and microbiological identification

For this study, 34 samples collected from the external auditory canal of dogs diagnosed with canine otitis externa were included. Initially, all samples were recultured, purified, and identified for subsequent assays. The samples were streaked using the exhaustion technique on Blood Agar plates (5% sheep blood) and incubated aerobically at 35 °C ± 1 °C for up to 72 h. Evaluations were performed at 24, 48, and 72 h to determine growth patterns and to assess the morphotintorial and biochemical characteristics of the predominant colony-forming units (CFUs) (Quinn, 2005).

Isolates identified as Gram-positive, specifically coagulase-negative *Staphylococcus* spp. and *Corynebacterium* spp., were included in subsequent analyses, whereas Gram-negative isolates were excluded due to the well-known restricted spectrum of activity of nisin (Mattick; Hirsch; Berridge, 1947). *Corynebacterium* spp. were identified as pleomorphic, catalase-positive, and oxidase-negative Gram-positive bacilli. *Staphylococcus* coagulase negative were identified as catalase-positive Gram-positive cocci with pink colonies on mannitol salt agar (QUINN, 2005).

At the end of the identification process, the selected bacteria were stored in a freezing medium consisting of tryptic soy broth (TSB) (Kasvi®, Pinhais, Paraná, Brazil) supplemented with 10% glycerol (v/v) and maintained at −80 °C for further analyses (ATCC Bacteriology Culture Guide, 2024).

2.2 SISGEN registration

The study was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under registration number AF10531, in compliance with Law No. 13,123/2015 and Decree No. 8,772/2016.

2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility of all isolates was evaluated using the disk diffusion method. From a pure culture, two to three planktonic colonies were suspended in 0.9% saline solution until reaching a turbidity equivalent to 0.5 on the McFarland scale, corresponding to approximately 1×10^8 CFU/mL (CLSI, 2015). The suspensions were then inoculated onto the entire surface of Mueller–Hinton agar (MHA) plates (Kasvi®, Pinhais, Paraná, Brazil) using sterile swabs, followed by the placement of disks containing antimicrobials commonly used in the treatment of canine otitis externa (CLSI, 2023).

Plates were incubated aerobically at 35 °C ± 1 °C for 24 h. Inhibition zones were measured using a ruler, and diameters (in millimeters) were interpreted according to the breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2023). In the absence of veterinary-specific breakpoints, CLSI M100 human breakpoints were used exclusively for comparative purposes.

For *Corynebacterium* spp., plates were supplemented with 5% sheep blood and incubated for 18 h. The results were interpreted according to breakpoints established by the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST, 2025) or, when genus-specific breakpoints were unavailable, compared with criteria for phylogenetically related Gram-positive microorganisms, with results considered descriptive. The same antimicrobial panel was maintained to ensure methodological consistency and allow comparative analysis among the different bacterial genders isolated from canine otitis, as well as to reflect therapeutic practices commonly employed in routine veterinary care. All adopted values are presented in Table 1.

Table 1 – Antimicrobials selected for disk diffusion for coagulase-negative *Staphylococcus* spp. and *Corynebacterium* spp.

Antimicrobial:	Purpose	Disk (µg):	Sensitive (S):	Intermediate (I):	Resistant (R):	Reference:

Penicilin G	Prediction of β-lactamase production (*Staphylococcus* spp.)	1 U[ab]	≥ 29 mm[a] ≥ 50 mm[b]	–[a] / 12–49 mm[b]	≤ 28 mm[a] < 12 mm[b]	CLSI, 2023[a] BrCAST, 2025[b]
Oxacilin (OXA)	Prediction of methicillin resistance (mecA)	1 µg [ab]	≥ 18 mm [ab]	– [ab]	≤ 17 mm [ab]	CLSI, 2023[a]
Clindamicin (CLI)	Primary use, independent of β-lactamase.	2 µg	≥ 21 mm[a] ≥ 20 mm[b]	15–20 mm[a]	≤ 14 mm[a] < 20 mm[b]	CLSI, 2023[a] BrCAST, 2025[b]
Gentamicin (GEN)	Primary use	10 µg	≥ 15 mm[ab]	13–14 mm[ab]	≤ 12 mm[ab]	CLSI, 2023[a] CLSI, 2023[b]
Chloramphenicol (CLO)	Alternative or supplementary use	30 µg	≥ 18 mm[ab]	13–17 mm[ab]	≤ 12 mm[ab]	CLSI, 2023[a] CLSI, 2023[b]
Sulfamethoxazole Trimethoprim	Alternative or supplementary use	23,75/1,25 µg	≥ 16 mm[a] ≥ 23 mm[b]	11–15 mm[a]	≤ 10 mm[a] < 23 mm[b]	CLSI, 2023[a] BrCAST, 2025[b]
Enrofloxacin (EN)	Alternative or supplementary use	5 µg	≥ 23 mm[ab]	17–22 mm[ab]	≤ 16 mm[ab]	CLSI, 2023[a] CLSI, 2023[b]
Ciprofloxacin (CIP)	Comparative	5 µg	≥ 21 mm[a] ≥ 50 mm[b]	16–20 mm[a] 25–49 mm[b]	≤ 15 mm[a] < 25 mm[b]	CLSI, 2017[a] BrCAST, 2025[b]
Moxifloxacin (MFX)	Comparative	5 µg	≥ 21 mm[a] ≥ 25 mm[b]	16–20 mm[a]	≤ 15 mm[a] < 25 mm[b]	CLSI, 2017[a] BrCAST, 2025[b]

[a] Breakpoints applicable to coagulase-negativa *Staphylococcus* spp., interpreted according to CLSI criteria. [b] Breakpoints applicable to *Corynebacterium* spp., interpreted according to BrCAST, when available. In the absence of specific breakpoints for *Corynebacterium* spp., the results were considered descriptive.

2.4 Preparation of the nisin stock solution

To obtain a nisin stock solution at 40.000 IU/mL, 500 mg of nisin powder (2.5% purity; 1.000 IU/mg; Sigma-Aldrich©, St. Louis, Missouri, USA) were dissolved in 12.5 mL of 0.1 mol/L HCl (pH 2.0), and the volume was adjusted to 50 mL in a volumetric flask using sterile distilled water. From the nisin stock solution, 50 mL of a working solution at 1.000 IU/mL were

prepared by dilution in distilled water at a 1:40 ratio. The final solution was stored under refrigeration at 2-8 °C until further use.

2.5 Minimum inhibitory concentration (MIC)

All isolates were subjected to MIC determination using the broth microdilution technique, performed in triplicate. Initially, colonies from a pure culture grown on MHA were inoculated into Brain Heart Infusion (BHI) broth and incubated aerobically at 35 °C ± 1 °C for 24 h. Subsequently, broth turbidity was standardized to an absorbance of 0.08-0.13, corresponding to approximately 1×10^8 CFU/mL (CLSI, 2015), using a UV-Vis spectrophotometer (Thermo Scientific Evolution©, Waltham, Massachusetts, USA) at 625 nm (CLSI, 2015).

Sterile Mueller–Hinton broth (MHB) was added to each well of the columns of a 96-well microplate. Then, the nisin working solution was added to all wells of line A. Serial twofold dilutions were performed by transferring half of the mixture from each well to the subsequent well, resulting in eight distinct nisin concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.8125, and 3.90625 IU/mL), and a final volume of 200 µL. Finally, the standardized bacterial inoculum were added in triplicate to all wells, and the plate was incubated aerobically at 35 °C ± 1 °C for 24 h (CLSI, 2015).

To correct the effect of the solvent on bacterial growth, the same protocol was performed with nisin replaced by HCl diluted at the same concentration, constituting the solvent control. A negative control (NC, containing only MHB) and a positive control (PC, containing MHB and the standardized bacterial inoculum) were also included. The minimum inhibitory concentration (MIC) was determined based on the difference in optical density (OD) between wells containing nisin and their respective solvent control wells. MIC_{90} and MIC_{50} values were defined as the lowest antimicrobial concentrations capable of inhibiting 90% and 50% of the isolates, respectively (CLSI, 2015).

After OD reading, 15 µL of 0.01% resazurin solution (R7017, Sigma-Aldrich©, Darmstadt, Germany) were added to each well of the microplate, followed by an additional 1 h incubation under the same previous conditions. The reduction of resazurin to resorufin, indicative of active cellular metabolism, was evidenced by a color change from dark blue with low fluorescence to fluorescent pink. The MIC was defined as the concentration present in the first blue well of each row, from line H to line A (Kumar; Nagarajan; Uchil, 2018).

2.6 Minimum bactericidal concentration (MBC)

All isolates were subjected to MBC determination by plate growth evaluation. Aliquots of 10 µL from the suspensions corresponding to the MIC, as well as from one immediately higher dilution and two lower dilutions, were streaked onto Mueller–Hinton agar (MHA) plates using the simple streaking technique. Plates were incubated aerobically at 35 °C ± 1 °C for 24 h, and the minimum bactericidal concentration (MBC) was defined as the lowest concentration at which no visible bacterial growth was observed on the plate.

MBC_{90} and MBC_{50} were defined as the concentrations capable of killing 90% and 50% of the isolates, respectively (CLSI, 1999). The MBC/MIC ratio was used to infer the bactericidal nature of nisin, with values ≤ 4 considered indicative of bactericidal activity (Pankey; Sabath, 2004).

2.7 Growth curve and time-kill assay

Three isolates identified as coagulase-negative *Staphylococcus* spp. were selected for growth curve and time-kill assays. Colonies from pure cultures grown on MHA were inoculated in triplicate into 10 mL of MHB and incubated aerobically at 35 °C ± 1 °C for 24 h. After incubation, cultures were standardized using the same procedure described for MIC determination, and the standardized inoculum was supplemented with 100 µL of nisin at the MIC concentration, corresponding to the initial time point (T0). Cultures were incubated under the same environmental conditions, and OD was measured at 600 nm at 0, 3, 6, 9, 12, 24, and 48 h after inoculation. Based on the mean OD values obtained throughout the experimental period, bacterial growth curves were constructed, allowing the evaluation of antimicrobial activity and the persistence of nisin action over time (He *et al*., 2009).

For assessment of cell viability, 10 µL aliquots were plated onto MHA after each time-point reading, enabling the inference of cellular viability and bacterial survival dynamics over time. The reduction in bacterial viability was determined by the difference between the number of viable cells at time zero and at subsequent time points. Reductions of less than 3 $\log_{10}$ CFU/mL were interpreted as bacteriostatic or time-dependent bactericidal effects (Messick; Rodvold; Pendland, 1999). For both assays, positive (MHB with standardized inoculum) and negative (MHB only) controls were included. To correct the solvent effect, the same protocol was performed with nisin replaced by HCl diluted at the same concentration.

2.8 Biofilm formation, inhibition (MBIC), and eradication (MBEC)

Initially, the estimated degree of biofilm formation was assessed. Colonies from pure cultures of the same three isolates selected for the previous assays were inoculated into BHI broth and incubated under the same environmental conditions for 24 h. Subsequently, cultures were standardized in tryptic soy broth (TSB) supplemented with 1% glucose, using the same absorbance range and wavelength previously described. Aliquots of 200 µL of the standardized inoculum were dispensed into 96-well microplates and incubated under the same conditions and for the same duration (Stepanovic *et al*., 2000).

After incubation, the contents of the wells were discarded, and the plates were washed three times with 200 µL of 0.9% NaCl solution to remove non-adherent cells. The plates were then air-dried at room temperature. Subsequently, 200 µL of methanol were added to each well and left for 15 min to fix the biofilm. Methanol was removed, and the wells were stained with 200 µL of crystal violet for 15 min. After staining, the plates were washed three times with distilled water to remove excess dye. To resolubilize the adhered biofilm, 240 µL of glacial acetic acid were added to each well (Stepanovic *et al*., 2000).

Positive (*Staphylococcus aureus* ATCC 6538, a known biofilm producer) and negative (*Staphylococcus epidermidis* ATCC 12228) controls were included. OD was measured at 600 nm using a microplate reader (Multiskan FC, Thermo Scientific®, San Jose, California, USA), and isolates were categorized according to their biofilm-forming capacity (Stepanovic *et al*., 2000).

To evaluate the ability to inhibit biofilm formation, the assay described above was repeated with the addition of nisin at the concentration corresponding to the MIC. Microplates were incubated under the same experimental conditions previously described, and biofilm formation was quantified by measuring OD at the same wavelength. To assess the ability to eradicate previously formed biofilm, the assay was conducted in a similar manner, differing only in the timing of nisin addition, which occurred after 24 h of initial incubation of the isolates, corresponding to the mature biofilm phase. After reincubation under the same conditions, residual biofilm was quantified according to the method described previously (Stepanovic *et al*., 2000).

2.9 In vitro cellular toxicity assay

Toxicity was evaluated using a hemolysis assay. A 2% erythrocyte suspension was prepared in phosphate-buffered saline (PBS) from human peripheral blood collected from a study researcher and aliquoted in heparinized tubes (manufacturer). The suspension was centrifuged at 2.500 revolutions per minute (rpm) for 10 min to separate erythrocytes, which were subsequently washed three times with 0.9% NaCl solution to remove plasma residues. In microtubes, 100 µL of erythrocytes in saline solution were mixed with nisin at the highest MIC concentration observed among the tested isolates. The microtubes were incubated in a water bath at 35 °C for 1 h and then centrifuged for 5 min to separate non-hemolyzed erythrocytes (Da Paz *et al*., 2022).

The following controls were included: negative control (erythrocytes and 0.9% NaCl), positive control (erythrocytes and distilled water), and solvent control (erythrocytes and diluted HCl). After incubation, the supernatants were collected and OD was measured at 540 nm. Substances inducing 10% or more hemolysis were considered toxic (Zhang *et al*., 2019). The hemolysis rate was calculated as follows:

$$\text{Hemolysis rate (\%)} = (AT - ACN) \times 100 / (ACP - ACN)$$

Where:
AT = absorbance of the test sample
ACN = absorbance of the negative control
ACP = absorbance of the positive control

2.10 Statistical analysis

Results from antimicrobial susceptibility testing and biofilm formation assessment were expressed as absolute and relative frequencies (%). MIC and MBC values were analyzed descriptively and presented as minimum and maximum values, mean and standard deviation, median, and interquartile range, when applicable. Data related to biofilm formation and eradication were initially evaluated for normality and homogeneity of variances. Group comparisons were performed using one-way analysis of variance (ANOVA), with Welch's correction when necessary, followed by Tukey's post hoc test for multiple comparisons. Additionally, analyses were confirmed using the nonparametric Kruskal–Wallis test. Differences were considered statistically significant when $p < 0.05$.

3.3 RESULTS:

Corynebacterium spp. isolates (n = 24) exhibited high rates of *in vitro* susceptibility, with 100% sensitivity to clindamycin (n = 24), chloramphenicol (n = 24), gentamicin (n = 24), moxifloxacin (n = 24), and enrofloxacin (n = 24). Ciprofloxacin showed variable activity, with 75.0% of isolates classified as susceptible (n = 18), 12.5% as intermediate (n = 3), and 12.5% as resistant (n = 3). For sulfamethoxazole–trimethoprim, susceptibility was observed in 83.3% of the isolates (n = 20), while 16.7% were resistant (n = 4). In contrast, penicillin showed the poorest performance, with only 20.8% of isolates classified as susceptible (n = 5), 33.3% as intermediate (n = 8), and 45.8% as resistant (n = 11).

Among coagulase-negative *Staphylococcus* spp. isolates (n = 12), universal resistance to penicillin G was observed (100%; n = 12). Resistance to oxacillin was detected in 58.3% of the isolates (n = 7), while 41.7% were classified as susceptible (n = 5). For clindamycin, 75.0% of isolates were resistant (n = 9), 8.3% intermediate (n = 1), and only 16.7% susceptible (n = 2). Regarding gentamicin, 75.0% of isolates were susceptible (n = 9) and 25.0% were resistant (n = 3). For sulfamethoxazole–trimethoprim, 58.3% of isolates were susceptible (n = 7), 8.3% intermediate (n = 1), and 33.3% resistant (n = 4). Among fluoroquinolones, equivalent resistance was observed to enrofloxacin and ciprofloxacin (50.0%; n = 6 each), whereas moxifloxacin showed the highest activity within this group, with 91.7% of isolates classified as susceptible (n = 11) and only one resistant isolate (8.3%; n = 1).

The MIC of nisin showed wide variability among the evaluated isolates, with 82% of the *Corynebacterium* isolates (n = 18) and 25% of the coagulase-negative *Staphylococcus* spp. isolates (n = 3) showing some inhibition level. For the first, MIC values ranged from 15.62 to 500 IU/mL. It was not possible to establish MIC_{50} and MIC_{90} values, as the highest similarity of results occurred at the 250 IU/mL breakpoint in only 41.67% of the isolates (n = 10), indicating that most isolates required relatively high concentrations for growth inhibition. For the second, MIC values ranged from 62.5 to 250 IU/mL. However, 9 of the 12 wasn't been inhibited by the peptide at any of the concentrations tested, which precluded the determination of MIC_{50} and MIC_{90} values.

Bactericidal activity was observed in only two *Corynebacterium* spp. isolates, both presenting a MBC of 250 IU/mL. Due to the small number of isolates with measurable bactericidal effect, it was not possible to establish population-based MBC parameters, such as MBC_{50} or MBC_{90}, nor to perform robust comparative analyses among groups, such as MBC/MIC ratio. In addition, none of the coagulase-negative *Staphylococcus* spp. isolates

exhibited a measurable MBC under the tested conditions, indicating the absence of detectable bactericidal activity for this genus. The results are presented in Table 3.

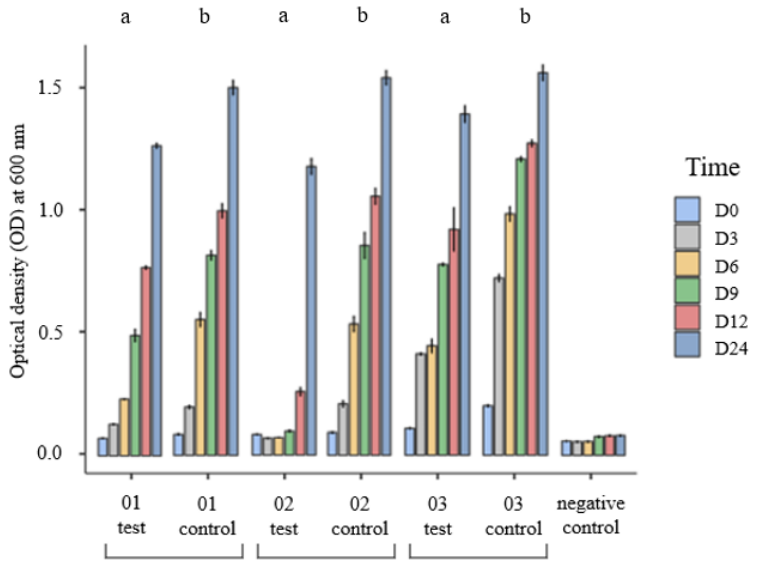
Table 3 - MIC and MBC of nisin on 24 strains of *Corynebacterium* spp. and 12 strains of coagulase negative *Staphylococcus* isolated from canine external otitis.

Bacteria:	Parameter:	Median + Interquartile range (IU/mL):	Average ± SD (IU/mL):
Corynebacterium spp.	MIC	50 (12.5 - 50.0)	36.4 ± 26.7
	MBC	50 (50 - 50)	50 ± 0.0
Staphylococcus spp.	MIC	12.5 (12.5 - 31.3)	25.0 ± 21.7
	MBC	NA*	NA*

MIC: minimum inhibitory concentration. MBC: minimum bactericidal concentration. IU: international units. SD: standard deviation. NA*: not applicable because most values were above the highest concentration tested (500 UI/mL), characterizing right-censored data. From: the author, 2026.

During the construction of the growth curve, nisin exerted a significant effect on bacterial growth over the evaluated period. Statistical analysis demonstrated a significant effect of incubation time (Kruskal–Wallis, $\chi^2 = 56.5$; $p < 0.001$), bacterial isolate (ANOVA, $F = 1012.3$; $p < 0.001$), and the interaction between time and bacteria (ANOVA, $F = 88.7$; $p < 0.001$). Initially (D0–D3), the three bacteria exhibited similar absorbance values. However, from D6 toD9, a progressive increase in absorbance was observed, indicating partial recovery, with statistically significant differences among the isolates. Between D12 and D24, all isolates showed a significant increase in absorbance ($p < 0.001$), as can be found in Figure 1.

Figure 1. Growth curve of coagulase-negative *Staphylococcus* spp. isolates exposed to nisin at MIC-equivalent concentration



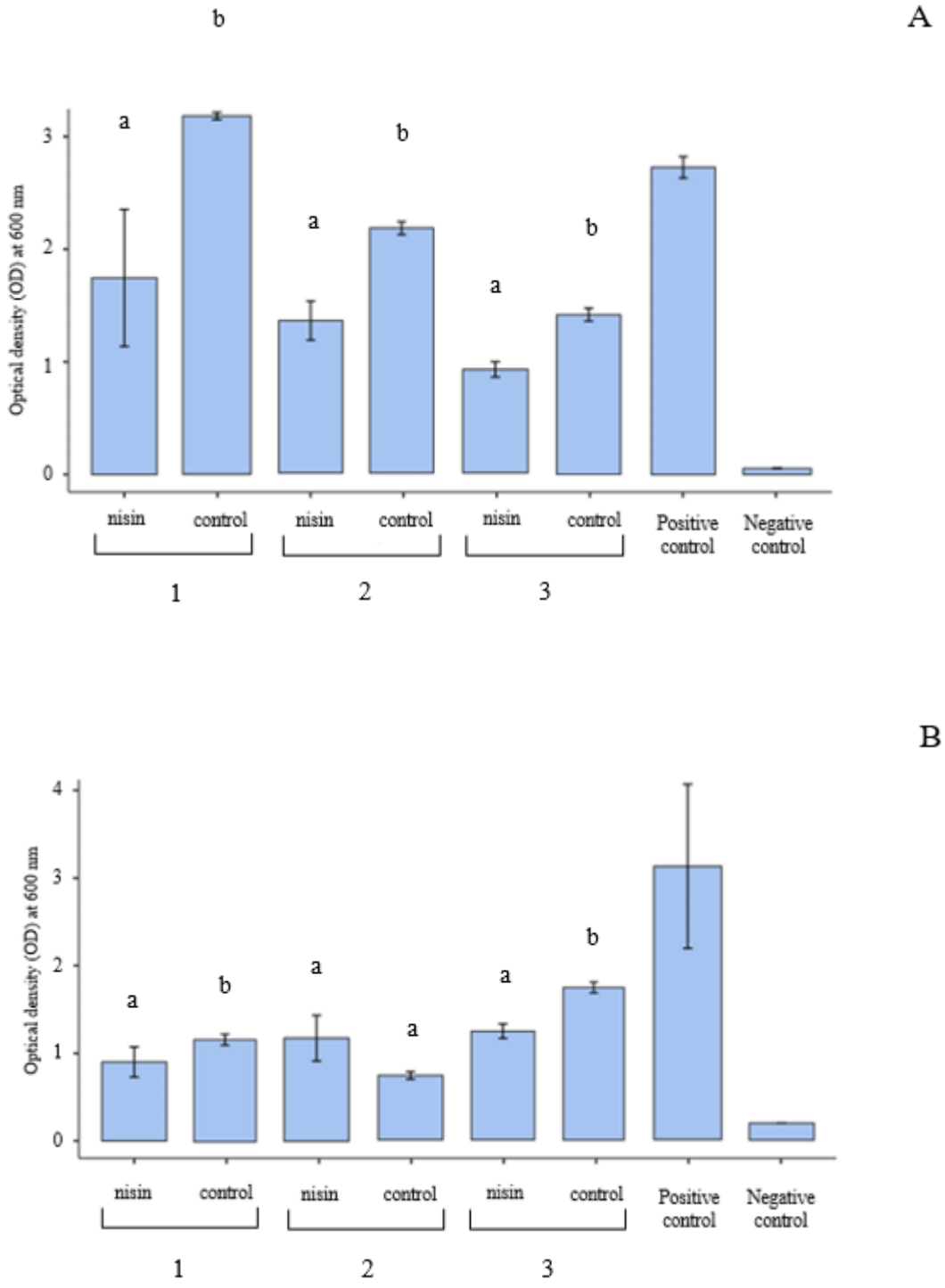
Different letters indicate a statistically significant difference in optical density (OD) between the control group and the test group in the same isolate ($p < 0.05$). From: the author, 2026.

In the time-kill assay, two of the three evaluated bacteria presented uncountable colony-forming unit (CFU) counts from the initial time point (T0), precluding the evaluation of logarithmic reductions in bacterial load over the analyzed period. For the third isolate, a count of 2.0×10^3 CFU/mL was observed at D0, followed by an increase to 1.22×10^4 CFU/mL at D3, indicating bacterial survival and multiplication even during the early phases.

Regarding biofilm formation, 20.6% (7/34) of the total isolates were identified as biofilm producers. The 22 *Corynebacterium* spp. were classified as non-biofilm producers. In relation to coagulase-negative *Staphylococcus* spp., 58.3% (7/12) were classified as biofilm producers. Among these last isolates, 28.6% (2/7) were weak producers, 28.6% (2/7) were moderate producers, and 42.8% (3/7) were strong producers. In the moderately and strongly producing isolates, treatment with nisin promoted a significant reduction in biofilm formation compared to the positive control (Welch's ANOVA, $p < 0.001$; Kruskal–Wallis, $p = 0.005$), whereas in weak biofilm producers only partial reduction was observed, as shown in Figure 2A.

Concerning biofilm eradication, statistically significant differences among groups were observed (Kruskal–Wallis, $p = 0.012$). However, biomass reduction was limited, and complete biofilm eradication was not observed for any of the evaluated bacteria, as illustrated in Figure 2B. These findings indicate that nisin is more effective in preventing biofilm formation than in removing previously established biofilms.

Figure 2 - Effect of nisin (b) compared to control (a) against biofilm formation (A) and erradication (B) of 3 strains of coagulase negative *Staphylococcus* spp. isolated from canine otitis externa.



C+: positive control. C-: negative control. Different letters indicate a statistically significant difference in optical density (OD) between the control group and the test group in the same isolate ($p < 0.05$). From: the author, 2026.

Finally, the hemolysis assay indicated that nisin was non-toxic, presenting an estimated hemolysis index of 0.20% compared with the control group.

3.4 DISCUSSION

Corynebacterium spp. isolates were generally susceptible to most antimicrobials, except penicillin, while coagulase-negative *Staphylococcus* spp. showed high resistance, particularly to penicillin G, oxacillin, and clindamycin. Nisin exhibited mainly bacteriostatic activity, with limited bactericidal effect, and effectively reduced biofilm formation in moderate and strong coagulase-negative *Staphylococcus* spp. producers, but had low efficacy against established biofilms. Nisin was non-toxic in the hemolysis assay.

Recent literature addressing *Corynebacterium* in the context of canine otitis remains scarce. Available studies suggest that this genus is commonly detected in polymicrobial infections, although its isolated pathogenic role appears limited. Consistent with these reports, the isolates evaluated in the present study exhibited comparatively low antimicrobial resistance rates when contrasted with other otitis-associated pathogens (Henneveld *et al*., 2012; Garcias *et al*., 2025). Furthermore, the absence of biofilm formation observed corroborates the hypothesis that *Corynebacterium* spp. primarily are opportunistic or commensal microrganisms within the auditory canal. Nevertheless, their clinical relevance should be considered inthe context of the overall microbial community, as their presence in polymicrobial infections may modify local microbial interactions and ultimately influence therapeutic outcomes in canine otitis externa.

The involvement of coagulase-negative *Staphylococcus* spp. species in canine otitis externa has been consistently associated with heterogeneous and frequently unfavorable antimicrobial resistance profiles. Recent studies have reported species such as *S. chromogenes*, *S. simulans* and *S. lentus* exhibiting resistance to multiple antimicrobial classes, particularly β-lactams and lincosamides, a pattern also observed in the present study (Lee *et al*., 2019; Dégi *et al*., 2024; Núñez *et al*., 2025). The elevated frequency of oxacillin resistance suggests the possible presence of methicillin-resistant coagulase-negative staphylococci (MR-CoNS), a clinically relevant finding due to their frequent association with multidrug resistance phenotypes. Moreover, the universal resistance to penicillin G probably reflects extensive prior exposure to β-lactams, wich raising concerns about empirical therapeutic strategies in canine

otitis externa. In contrast, the marked susceptibility to moxifloxacin aligns with reports indicating superior *in vitro* activity of later-generation fluoroquinolones against this group of pathogens, although it is not yet licensed for veterinary use in Brazil (Popa *et al*., 2025).

To our knowledge, no studies have evaluated the activity of nisin against bacteria associated with canine otitis externa. Investigations involving other clinically relevant Gram-positive pathogens, such as *Streptococcus suis* and Enterococcus spp., reported MIC values ranging from 0.12 to 4.0 µg/mL and from 4.10 to 19.25 µg/mL, as well as MBC values ranging from 0.25 to 8 µg/mL and from 18.57 to >100 µg/mL, respectively (Cunha *et al*., 2020; Zhu *et al.,* 2021). In the former study, nisin exhibited bactericidal activity against *S. suis* at concentrations ≥ 2× MIC, witch was considered, according to the literature, as a bactericidal effect (Cunha *et al*., 2020).

These findings, which contrast with the results of the present study, may be attributed to structural characteristics of the bacterial cell wall, such as the presence of mycolic acids in *Corynebacterium* spp., which act as a barrier to antimicrobial peptides, as well as to adaptive responses typical of bacteria persisting in chronic inflammatory environments. In our study, the absence of a ≥ 3 $\log_{10}$ reduction in CFU/mL, together with the recovery of bacterial growth observed in the growth curve, indicates that under the tested conditions and at MIC-equivalent concentration, nisin exerted a mainly bacteriostatic and transient inhibitory effect.

The ability to nisin inhibit biofilm formation is consistent with previous reports demonstrating antibiofilm activity during the early stages of bacterial adhesion and maturation. Studies involving *S. aureus* have shown that subinhibitory concentrations of nisin significantly reduce extracellular matrix production and modulate the expression of virulence factors (Ganguly *et al*., 2025). In addition, previous findings have demonstrated that nisin reduces biofilm biomass in a dose-dependent manner, achieving inhibition rates of up to approximately 90% at the MIC concentration, while lower concentrations result in partial, but significant inhibition (Sharafi *et al*., 2024).

Regarding the eradication of previously established biofilms, the limited efficacy observed in this study aligns with reports showing that although nisin is capable of penetrating biofilm matrices, it results in substantially lower reductions in bacterial viability in mature biofilms compared to planktonic cells (Godoy-Santos *et al*., 2019). Experimental data indicate that eradication of mature biofilms often requires considerably higher concentrations, with MBEC values ranging from 2048 to 4096 µg/mL, highlighting the intrinsic tolerance of sessile communities (Sharafi *et al*., 2024). Taken together, these data suggest that nisin affects biofilms more efficiently as a preventive rather than a therapeutic agent. Additionally, synergistic

interactions between nisin and β-lactam antibiotics, such as oxacillin, have been reported, resulting in significant reductions in MBEC values and enhanced biofilm disruption (Sharafi *et al.,* 2024). These observations indicate that combination strategies may help overcome the limited efficacy observed against mature biofilms and justify further investigation in the context of canine otitis externa.

Finally, available evidence regarding the evaluation of nisin toxicity through hemolysis assays has reported absent or minimal hemolysis at concentrations below 50 µg/mL, whereas exposure to concentrations approaching 400 µg/mL resulted in nearly complete hemolysis, suggesting loss of cellular selectivity at higher doses (Soltani *et al.,* 2022). Similarly, others observed low hemolytic activity, with relative hemolysis values of approximately 6.6% at 40 µM (~134 µg/mL) (Paiva *et al*., 2012). Although such data are not derived from ear-specific experimental models, they provide important safety parameters for interpreting the present findings.

This study has some limitations. The analyzed samples corresponded to a reduced sample size, originated from different laboratory collections, and were not stratified according to the animals' previous therapeutic history, which may have influenced the microbiological profile and susceptibility patterns observed. Furthermore, the absence of cell culture assays prevents accurate inference regarding the safety of nisin for topical use in the auditory environment. Therefore, the results provide relevant initial evidence of the antimicrobial and antibiofilm potential of nisin in the context of canine otitis externa, reinforcing the need for further in vivo studies to better elucidate its clinical applicability.

3.5 CONCLUSION

This study provides the initial *in vitro* evaluation of nisin against bacteria associated with canine otitis externa. Nisin demonstrated antimicrobial activity, with a predominantly bacteriostatic and transient effect at MIC-equivalent concentrations. It significantly inhibited biofilm formation but showed limited efficacy against established biofilms, indicating greater potential as a preventive rather than eradicative agent. Despite these promising findings, clinical extrapolation remains limited. Further studies incorporating expanded panels of isolated microorganisms, optimized dosing strategies, combination approaches, and ear-specific cytotoxicity studies and *in vivo* models are required to define nisin therapeutic applicability in canine otitis externa.

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4 SEGUNDO EXPERIMENTO

EVALUATION OF THE TOXICITY, ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY OF NISIN AGAINST *Staphylococcus aureus* ISOLATED FROM BOVINE MASTITIS:

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RESUMO

A mastite bovina é a enfermidade mais frequente e economicamente relevante da bovinocultura leiteira. *S. aureus* assume papel central devido à sua capacidade de desenvolver resistência antimicrobiana e formar biofilmes na glândula mamária. O objetivo do presente estudo foi avaliar a atividade antimicrobiana e antibiofilme da nisina frente a isolados de *S. aureus*

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provenientes de mastite bovina. Foram incluídos 25 isolados obtidos de amostras de leite com suspeita de mastite. O perfil de suscetibilidade antimicrobiana foi determinado pelo método de difusão em disco, enquanto a atividade da nisina foi avaliada por meio da determinação da concentração inibitória mínima e concentração bactericida mínima. A atividade antibiofilme foi analisada por ensaio de coloração com cristal violeta. Além disso, foi avaliada a toxicidade da nisina pelo teste de hemólise em eritrócitos. Os resultados demonstraram elevados índices de resistência a antimicrobianos β-lactâmicos. A nisina apresentou atividade antimicrobiana *in vitro* frente aos isolados avaliados, com variação nas concentrações necessárias para inibição bacteriana, possivelmente associada à heterogeneidade fenotípica das cepas. Observou-se inibição da formação de biofilme e redução discreta na erradicação de biofilmes já formados. Em concentrações inibitórias, a nisina foi considerada não hemolítica. Conclui-se que a nisina apresenta ausência de toxicidade em células eucariontes, bem como atividades antimicrobiana e antibiofilme *in vitro* promissoras frente a isolados de *S. aureus* associados à mastite bovina.

Palavras-chave: resistência antimicrobiana, peptídeos, bovinocultura leiteira, sanidade animal, inflamação da glândula mamária.

ABSTRACT

Bovine mastitis is the most frequent and economically relevant disease in dairy cattle farming. *S. aureus* plays a central role due to its ability to develop antimicrobial resistance and form biofilms in the mammary gland. The objective of this study was to evaluate the antimicrobial and antibiofilm activity of nisin against *S. aureus* isolates from bovine mastitis. Twenty-five isolates obtained from milk samples suspected of mastitis were included. The antimicrobial susceptibility profile was determined by the disk diffusion method, while nisin activity was evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Antibiofilm activity was analyzed by crystal violet staining assay. In addition, nisin toxicity was evaluated by the erythrocyte hemolysis test. The results demonstrated high resistance rates to β-lactam antimicrobials. Nisin showed in vitro antimicrobial activity against the evaluated isolates, with variation in the concentrations required for bacterial inhibition and death, possibly associated with the phenotypic heterogeneity of the strains. Inhibition of biofilm formation and a slight reduction in the eradication of already formed biofilms were observed. At inhibitory concentrations, nisin was

considered non-hemolytic. It is concluded that nisin presents an absence of toxicity in eukaryotic cells, as well as promising in vitro antimicrobial and antibiofilm activities against *S. aureus* isolates associated with bovine mastitis.

Keywords: antimicrobial resistance; peptides; dairy cattle farming; animal sanity, mammary gland inflamation.

4.1 INTRODUCTION

According to data from the Brazilian Institute of Geography and Statistics (IBGE), Brazilian milk production reached approximately 35.7 billion liters in 2024, consolidating itself as one of the main economic and social pillars of national agribusiness. In this context, bovine mastitis, characterized as an inflammatory process of the mammary gland (Sharun *et al.,* 2021), stands out as the most frequent and economically relevant disease in dairy farming due to its direct negative impact on milk yield, composition, and quality (Gomes; Henriques, 2016), as well as indirect costs related to veterinary assistance, antimicrobial use, and the disposal of milk containing residues (Zhylkaidar *et al*., 2021; Caneschi *et al*., 2023).

Among the etiological agents, *S. aureus* plays a central role in bovine mastitis due to its high capacity to acquire antimicrobial resistance and to form biofilms within the mammary gland, characteristics that favor intramammary persistence, recurrence of infections, and progression to chronic conditions (Bellato *et al.,* 2023; Suthovski *et al*., 2023; Demontier *et al.,* 2025). In light of these challenges, alternative compounds to conventional antimicrobials, such as antimicrobial peptides, have been widely investigated due to their therapeutic potential against planktonic cells and biofilm-associated structures (Ceotto-Vigoder *et al*., 2016).

Nisin is a lantibiotic bacteriocin typically produced by *Lactococcus lactis*, whose antimicrobial activity is associated with the inhibition of bacterial cell wall synthesis. This peptide exhibits activity against a wide range of Gram-positive bacteria, with evidence of action against both planktonic cells and sessile forms associated with biofilm formation (Małaczewska; Kaczorek-Łukowska, 2021). Thus, the aim of the present study was to evaluate the toxic, antimicrobial, and antibiofilm activity of nisin against *S. aureus* isolates obtained from bovine mastitis, aiming to contribute to the development of more effective therapeutic strategies for the control of this disease.

4.2 MATERIAL AND METHODS

2.1 Isolates:

A total of 25 isolates compatible with *S. aureus* obtained from milk samples with suspected bovine mastitis were included. Bacteria compatible with other bacterial genera were excluded. Eligible isolates were preserved in a freezing medium consisting of tryptic soy broth (TSB) (Kasvi®, Pinhais, Paraná, Brazil) supplemented with 10% glycerol (v/v) and stored at −80 °C for subsequent analyses.

2.2 Registration in Sisgen:

The study was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN), under registration number AF10531, in accordance with Law No. 13.123/2015 and Decree No. 8.772/2016.

2.3 Preparation of the nisin stock solution:

To obtain a nisin stock solution at 40.000 IU/mL, 500 mg of nisin powder (2.5% purity; 1.000 IU/mg; Sigma-Aldrich©, St. Louis, Missouri, USA) were dissolved in 12.5 mL of 0.1 mol/L HCl (pH 2.0), and the volume was adjusted to 50 mL in a volumetric flask with sterile distilled water. Subsequently, the nisin stock solution was diluted in sterile distilled water to achieve initial concentrations of 10.000 IU/mL, 4.000 IU/mL and 1.000 IU/mL.

2.3 Disk diffusion:

Antimicrobial susceptibility testing of the samples was performed using the disk diffusion technique. The number of isolates tested varied according to the availability of each antimicrobial agent. From a pure culture, two to three planktonic colonies were suspended in 0.9% saline solution until a 0.5 McFarland standard was achieved, corresponding to 1×10^8 CFU/mL (CLSI, 2015). The suspensions were then inoculated over the entire surface of plates containing Mueller–Hinton agar (MHA) (Kasvi®, Pinhais, Paraná, Brazil) using a sterile swab, and disks containing antimicrobial agents commonly used in the treatment of bovine mastitis were applied, according to material availability in the laboratory. Plates were incubated under aerobic conditions at 35 °C ± 1 °C for 24 h. Inhibition zones were measured using a ruler, and diameters in millimeters were interpreted according to the breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2023), as described in Table 1.

Table 1 – Antimicrobials selected for disk diffusion for *Staphylococcus aureus* isolated from bovine mastitis.

Antimicrobial:	Disk (µg):	Sensitive (S):	Intermediate (I):	Resistant (R):	Reference:
Oxacilin	1	≥ 13 mm	11–12 mm	≤ 10 mm	CLSI, 2023
Penicilin	10 UI	≥ 29 mm	-	≤ 28 mm	CLSI, 2023
Ampicilin	10	≥ 29 mm	-	≤ 28 mm	CLSI, 2023
Tetracycline	30	≥ 19 mm	15–18 mm	≤ 14 mm	CLSI, 2023
Enrofloxacin	5	≥ 21 mm	17–20 mm	≤ 16 mm	CLSI, 2023
Gentamicin	10	≥ 15 mm	13–14 mm	≤ 12 mm	CLSI, 2023
Ciprofloxacin	5	≥ 21 mm	16–20 mm	≤ 15 mm	CLSI, 2023
Sulfamethoxazole Trimethoprim	23,75/1,25 µg	≥ 16 mm	11–15 mm	≤ 10 mm	CLSI, 2023
Erythromycin	15	≥ 23 mm	14–22 mm	≤ 13 mm	CLSI, 2023

From: the author, 2026.

2.4 Minimal Inhibitory Concentration (MIC):

To evaluate nisin antimicrobial effect, all samples were subjected to this assay using the broth microdilution technique, performed in triplicate. Initially, colonies from a pure culture grown on MHA were inoculated into Brain Heart Infusion (BHI) broth and incubated under aerobic conditions at 35 °C ± 1 °C for 24 h. Subsequently, the turbidity of the cultures was standardized to 0.08–0.13 Å, corresponding to 1×10^8 CFU/mL (CLSI, 2015), using a UV–Vis spectrophotometer (Thermo Scientific Evolution©, Waltham, Massachusetts, USA) at 625 nm (CLSI, 2015).

Sterile Mueller–Hinton broth (MHB) was added to each well of the columns of a 96-well microplate. Serial microdilutions were performed using initial solutions of 10.000 IU/mL and 4000 IU/mL of nisin, resulting in final concentrations ranging from 5000 to 78.12 IU/mL and from 2000 to 62.5 IU/mL, respectively, resulting in a final volume of 200 µL. Finally, the standardized bacterial inoculum was added in triplicate to all wells, and the plate was incubated under aerobic conditions at 35 °C ± 1 °C for 24 h. Negative control wells (NC, containing only MHB) and positive control wells (PC, containing MHB and the standardized bacterial inoculum) were included. MIC_{90} and MIC_{50} values were defined as the lowest antimicrobial concentrations capable of inhibiting 90% and 50% of the isolates, respectively (CLSI, 2015).

After incubation, 15 µL of 0.01% resazurin solution (R7017, Sigma-Aldrich©, Darmstadt, Germany) was added to each well of the microplate, followed by an additional incubation for 1 h under aerobic conditions at 35 °C ± 1 °C. The reduction of resazurin to

resorufin was indicated by a color change from dark blue with low fluorescence to fluorescent pink, and the minimum inhibitory concentration was defined as the concentration present in the first blue well of each row. (Kumar; Nagarajan; Uchil, 2018).

2.5 Minimum bactericidal concentration (MBC) and MBC/MIC ratio:

All samples were subjected to this assay by evaluating bacterial growth on agar plates. Aliquots of 10 µL from the suspensions corresponding to the MIC, one immediately higher dilution, and two lower dilutions were streaked onto plates containing MHA using the simple streaking technique. The plates were incubated under aerobic conditions at 35 °C ± 1 °C for 24 h, and the minimum bactericidal concentration (MBC) was defined as the lowest concentration at which no visible bacterial growth was observed on the agar plates. MBC_{90} and MBC_{50} values were defined as the lowest antimicrobial concentrations capable of killing 90% and 50% of the isolates, respectively (CLSI, 1999). The MBC/MIC ratio was used to infer the bactericidal character of nisin, considering values ≤4 as indicative of bactericidal activity (Pankey; Sabath, 2004).

2.6 Biofilm formation, inhibition (MBIC), and eradication (MBEC):

Initially, the estimated degree of biofilm formation was assessed. Colonies from pure cultures of the same three bacteria selected for the previous assays were inoculated into BHI broth and incubated under the same environmental conditions for 24 h. Subsequently, the cultures were standardized in TSB broth supplemented with 1% glucose to the same absorbance range and wavelength previously described. Aliquots of 60 µL of the standardized inoculum were pipetted into 96-well microplates and incubated under the same conditions and for the same incubation period (Stepanovic *et al.*, 2000).

After the incubation period, the contents of the wells were discarded, and the plates were washed three times with 200 µL of 0.9% NaCl solution to remove non-adherent cells. The plates were then allowed to air-dry at room temperature. Subsequently, 200 µL of methanol was added to each well and left for 15 min to fix the biofilm. The methanol was removed, and the wells were stained with 200 µL of crystal violet for 15 min. After staining, the plates were washed three times with distilled water to remove excess dye. To resolubilize the adhered biofilm, 240 µL of glacial acetic acid was added to each well (Stepanovic *et al*., 2000).

The following controls were included: a positive control (*S. aureus* ATCC 6538, a known biofilm producer) and a negative control (*Staphylococcus epidermidis* ATCC 12228). Optical density (OD) readings were performed at 600 nm using a microplate reader (Multiskan

FC, Thermo Scientific®, San Jose, California, USA), and the isolates were categorized according to their biofilm-forming capacity as described in Table 2 (Stepanovic *et al*., 2000).

To assess the ability of nisin to inhibit biofilm formation, the assay described above was repeated with the addition of nisin at the concentration corresponding to the MIC in all bacteria classified as strongly biofilm-forming. The microplates were incubated under the same experimental conditions previously described, and biofilm formation was quantified by reading the optical density at the same wavelength. To assess the ability to eradicate previously formed biofilm, the assay was conducted similarly, differing in the timing of nisin addition, which occurred after 24 hours of initial incubation of the isolates, corresponding to the mature biofilm phase. After re-incubation under the same conditions, the residual biofilm was quantified according to the method described above (Stepanovic *et al*., 2000).

2.7 In vitro toxicity test:

The toxicity assessment was performed using the hemolysis assay. A 2% red blood cell suspension in phosphate-buffered saline (PBS) was prepared from human peripheral blood collected in a tube containing heparin. The suspension was centrifuged at 2500 repetitions per minute (rpm) for 10 minutes to separate the red blood cells, which were subsequently washed three times with 0.9% NaCl physiological saline solution to remove plasma debris. In a microtube, 100 µL of red blood cells were added to saline solution and nisin at the concentration corresponding to the highest MIC among the isolates tested. The microtubes were left in a water bath at 35 ºC for 1 hour and centrifuged for 5 minutes to separate the non-hemolyzed red blood cells (Da Paz *et al*., 2022).

The following controls were included: negative control (erythrocytes and 0.9% NaCl), positive control (erythrocytes and distilled water), and solvent control (erythrocytes and diluted HCl). Finally, the supernatants were removed, and the optical density was measured at 540 nm. A substance showing 10% or more hemolysis in the sample was considered toxic (Zhang *et al*., 2019). The hemolysis rate was calculated as follows:

$$\text{Hemolysis rate (\%)} = (TA - NCA) \times 100 / (PCA - NCA)$$

TA = absorbance of test sample

NCA = absorbance of negative control sample

PCA = absorbance of positive control sample

2.8 Statistical analysis

The results of antimicrobial susceptibility testing were expressed as absolute and relative frequencies (%). MIC and MBC values were analyzed descriptively and expressed as minimum, maximum, median, mean, and standard deviation. Biofilm formation data were analyzed using descriptive statistics based on absorbance values. For biofilm inhibition and eradication assays, comparisons between control and nisin-treated groups were performed using the paired Wilcoxon test. Differences were considered statistically significant when $p < 0.05$. Effect size was estimated using the r coefficient, calculated according to Rosenthal (1994) and interpreted based on Cohen (1988).

4.3 RESULTS

High resistance to β-lactam antibiotics was observed, as none of the tested isolates showed susceptibility to oxacillin (0%; 0/6), penicillin (0%; 0/18), or ampicillin (0%; 0/17). In contrast, high susceptibility rates were observed for fluoroquinolones and aminoglycosides. Susceptibility to enrofloxacin was detected in 91.3% of the isolates (21/23), while ciprofloxacin showed a susceptibility rate of 95.5% (21/22). For gentamicin, all tested isolates were classified as susceptible (100%; 18/18). Tetracycline exhibited 78.3% susceptible isolates (18/23), demonstrating relevant antimicrobial activity despite the presence of resistant isolates. For sulfamethoxazole–trimethoprim, susceptibility was observed in 52.4% of the isolates (11/21), indicating a heterogeneous response profile to this antimicrobial. Finally, regarding macrolides, erythromycin showed a low susceptibility rate, with only 18.2% of the isolates classified as susceptible (4/22).

All evaluated isolates were susceptible to nisin. MIC values ranged from 125 to 250 IU/mL, whereas MBC values ranged from 250 to 625 IU/mL. Among the analyzed isolates, 88% presented a MIC of 250 IU/mL, corresponding to the MIC50 value and approaching the MIC90, and 68% of the isolates presented a MBC of 500 IU/mL, correspondind to the MBC90. 100% of the isolates showed a MBC/MIC ratio ≤ 4, with an average of 1.94, indicating a bactericidal effect. The distribution of MIC and MBC values is presented in Table 3.

Table 3 - MIC and MBC of nisin on 25 strains of *S. aureus* isolated from bovine mastitis.

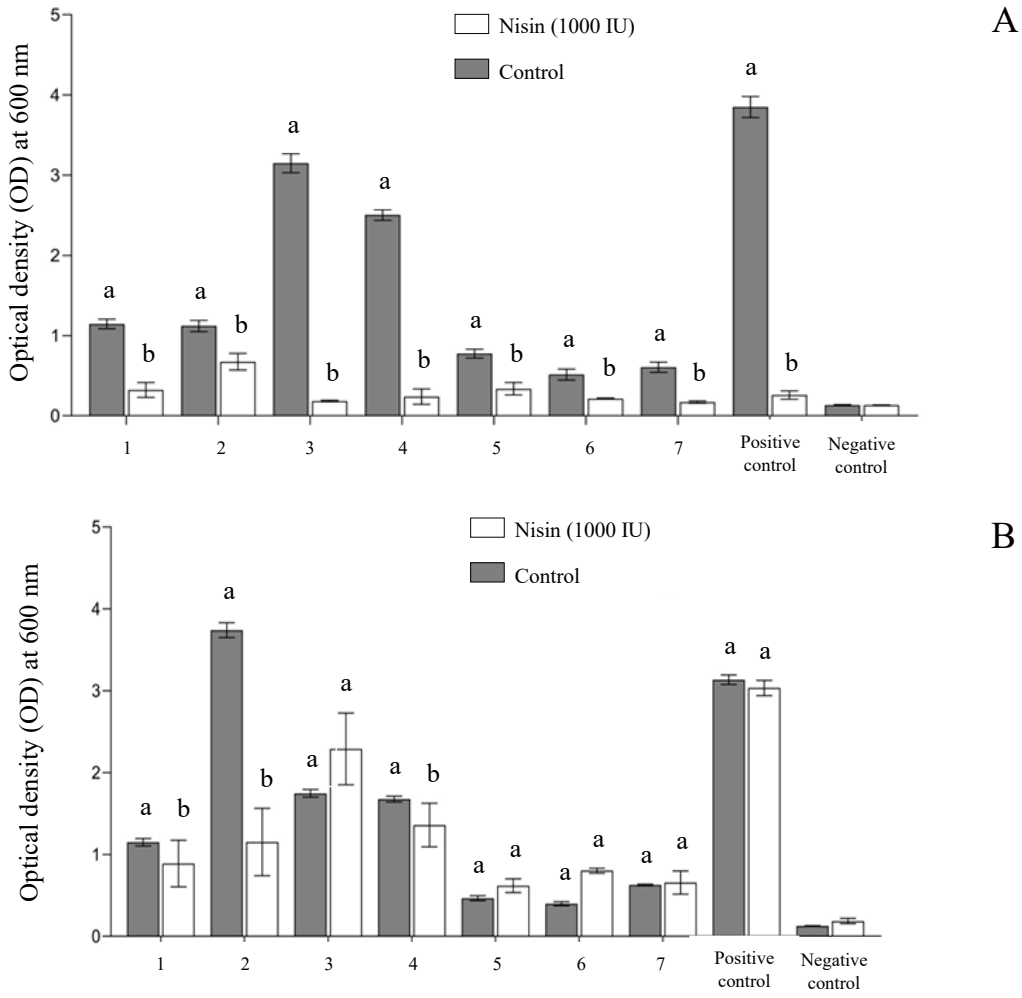
Parameter	Median + interquartile range (IU/mL)	Average ± SD (IU/mL)
MIC	250 (250-250)	238.74 ± 31.11

MBC	500 (500-500)	460.94 ± 110.29

MIC: minimum inhibitory concentration. MBC: minimum bactericidal concentration. IU: international units. SD: standard deviation. From: the autor, 2026.

Regarding biofilm formation, of the 25 isolates evaluated, 80% (n == 20) showed the ability to form biofilm, with 7 classified as strong biofilm producers, 4 as moderate producers, and 9 as weak producers. Figure 1A presents the comparison between strong biofilm-producing strains and the positive and negative controls, demonstrating that nisin promoted a marked reduction in biofilm production in all tested isolates. The presence of the peptide resulted in significantly lower OD values compared with the control group ($p < 0.0001$, $r = 0.63$), indicating its potential inhibitory effect on bacterial biofilm formation. In addition, in the eradication test, nisin showed a partial reduction on preformed biofilm over a 24 h period in 3 isolates ($p = 0.0395$), witch was considered a small effect ($r = 0.1$), and 4 showed greater biofilm formation in the presence of nisin, as shown in Figure 1B.

Figure 1 - Effect of nisin against biofilm formation (A) and erradication (B) of 7 strains of *S. aureus* isolated from bovine mastitis.



Different letters indicate a statistically significant difference in optical density (OD) between the control group and the test group in the same isolate ($p < 0.05$), while identical letters indicate that there was no statistically significant difference ($p \geq 0.05$). From: the autor, 2026.

Finally, in the hemolysis assay, nisin was considered non-toxic, with an estimated hemolysis index of 0.191%.

4.4 DISCUSSION:

The *S. Aureus* isolates showed high resistance to β-lactams, but were largely susceptible to fluoroquinolones, aminoglycosides, and tetracycline, while erythromycin and sulfamethoxazole–trimethoprim exhibited lower activity. All isolates were susceptible to nisin, which displayed bactericidal effects and effectively inhibited biofilm formation, although

eradication of preformed biofilms was limited. Nisin also showed no hemolytic activity, indicating its safety for potential therapeutic use.

The resistance profile observed in this study is consistent with previous reports for *S. aureus* isolated from bovine mastitis, particularly with regard to the high phenotypic resistance to β-lactams, which have been used as first-line treatment (Piaia *et al*., 2025). This form of resistance is frequently associated with β-lactamase production and clonal selection under therapeutic pressure (Beker; Demirbilek, 2025). National studies have demonstrated high rates of resistance to penicillin and ampicillin, while global analyses confirm penicillin as the antimicrobial with the highest prevalence of resistance in intramammary infections, with an increasing trend over time, especially in regions such as Latin America (Sánchez-Ceja *et al*., 2018; Molineri *et al*., 2021). In contrast, lower resistance rates have been described for aminoglycosides and fluoroquinolones, which is consistent with the high percentages of susceptibility observed in this study (Kawai *et al*., 2023). Despite the well-established antimicrobial and antibiofilm potential of nisin as a food preservative, studies that systematically evaluate its activity against clinical isolates associated with bovine mastitis are still scarce.

In quantitative terms, previous studies have demonstrated that nisin exhibits variable inhibitory activity against pathogens associated with bovine mastitis depending on the species analyzed. Assays involving *S. aureus* isolates reported minimum inhibitory concentration (MIC) values for wild-type nisin A around 1 to 2 µg/mL, whereas structural variants, such as nisin A M17Q, showed up to a fourfold increase in potency (Field *et al*., 2021). On the other hand, studies using commercial nisin reported MIC values ranging from 1 to 100 µg/mL for different mastitis-associated species, including *S. aureus, Streptococcus dysgalactiae*, and *Streptococcus uberis*. Minimum bactericidal concentration (MBC) values were consistently one to four times higher than the MICs, indicating bactericidal activity (Bennet *et al*., 2021). Another study reported variations between 78 and 624 IU/mL, with most isolates showing inhibition between 156 and 312 IU/mL (Kaczorek-Łukowska *et al*., 2025). Although the values obtained in the present study are slightly higher, this difference may be related to the phenotypic heterogeneity of clinical isolates and their prior exposure history to antimicrobials, factors that have been described as influencing variability in the response to nisin (Bennet *et al*., 2021).

Studies evaluating the action of nisin on *S. aureus* biofilms have demonstrated that its efficacy is strongly dependent on concentration, exposure time, and cell viability. In isolates associated with bovine mastitis, nisin showed limited ability to eradicate pre-formed biofilms, as observed in this study, resulting mainly in reduced cell viability, with limited effectiveness

in the structural eradication of the biofilm (Ceotto-Vigoder *et al*., 2016). Studies under continuous flow conditions and confocal microscopy analyses demonstrated that nisin is able to rapidly penetrate the biofilm matrix and cause damage to the cell membrane, although with a lower bactericidal effect compared to planktonic cells (Godoy-Santos *et al*., 2019). The increased biofilm biomass observed in wells exposed to nisin during biofilm formation may be explained by a stress-induced adaptive response at subinhibitory concentrations, as previously described by studies (Elawady *et al*., 2024). Conversely, when nisin was applied to pre-formed biofilms, no stimulatory effect was observed, likely due to the mature biofilm architecture and limited antimicrobial penetration.

Finally, the literature regarding the evaluation of nisin toxicity through hemolysis assays has demonstrated a clearly concentration-dependent effect. Absence or low levels of hemolysis were observed at concentrations below 50 µg/mL, whereas levels close to 400 µg/mL resulted in nearly complete hemolysis, indicating loss of cellular selectivity at high doses (Soltani *et al*., 2022). In another study, nisin exhibited low hemolytic activity, with relative hemolysis values of approximately 6.6% at a dose of 40 µM (~134 µg/mL) (Paiva *et al*., 2012). The results of this study, together with those reported in the literature, suggest that concentrations effective against bacteria can be achieved below levels associated with erythrocyte toxicity.

Additionally, a study evaluating the toxicity of nisin in bovine mammary epithelial cells indicated safety across all minimum inhibitory concentration ranges obtained, evidenced by cell viability above 50% (Santa Catarina *et al*., 2025). Previous studies have also identified nisin as an agent that promotes cell proliferation and increases cell lifespan (Sadrei *et al.*, 2022; Gao *et al*., 2022), besides presenting anti-apoptotic effect in human mesenchymal stem cells, reducing inflammatory damage and maintaining secretory function, which may favors mammary tissue recovery, although it may negatively interferes with the elimination of compromised cells. (Namjoo *et al*., 2022).

The present study has some limitations that should be considered when interpreting the results. The antimicrobial susceptibility profile was determined without the inclusion of cephalosporins, which represent one of the main classes used as first-line treatment for mastitis, limiting direct comparisons with therapeutic protocols widely employed in field conditions. Toxicity analysis was based solely on the hemolysis assay, indicating the need for additional studies to better characterize its safety. Therefore, future studies should include the evaluation of other relevant antimicrobial classes, combine phenotypic and genotypic analyses for a more comprehensive epidemiological understanding, and employ *in vivo* models in order to deepen knowledge regarding the efficacy and safety of nisin in the context of bovine mastitis.

4.5 CONCLUSION

This study demonstrated that nisin exhibits promising *in vitro* antimicrobial activity against *S. aureus* isolates associated with bovine mastitis, with a limited effect on pre-formed biofilms and a concentration-dependent toxicity profile. Further studies are needed to better define its potential applications.

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5 EXPERIMENTO ADICIONAL

5.1 OBTENÇÃO DE NANOESFERAS CONTENDO NISINA

As nanoesferas contendo nisina foram preparadas pelo método de emulsificação por ultrassonicação seguida de evaporação do solvente orgânico, utilizando poli(ε-caprolactona) (PCL) como polímero e álcool polivinílico (PVA) como agente estabilizante (Fessi *et al.*, 1989). O método consistiu na preparação de uma fase aquosa, composta por água destilada, nisina e PVA, e de uma fase orgânica (polimérica), constituída por PCL dissolvida em solvente orgânico.

Cinco formulações distintas (F1, F2, F3, F4 e F5) foram testadas, com o objetivo de avaliar a influência da composição das fases, dos solventes empregados, da concentração de polímero e da forma de incorporação da nisina sobre as características das nanoesferas. As variações entre as formulações estão descritas na Tabela 3.

Tabela 1 – Composição das formulações de nanoesferas de nisina.

Formulação	PCL (mg)	Solvente orgânico	PVA (mg)	Diluente aquoso	Forma e modo de incorporação da nisina
1	50	1,8 mL de diclorometano	200	100 mL de H_2O	10 µL na fase orgânica (solução em HCl)
2	100	20 mL de acetona	40	2,6 mL de H_2O + 1,4 mL de HCl 0,1 M	1 mg na fase aquosa (liofilizada)
3	300	80 mL de acetona	40	2,6 mL de H_2O + 1,4 mL de HCl 0,1 M	1 mg na fase aquosa (liofilizada)
4	50	1,8 mL de diclorometano	500	6,25 mL de HCl 0,1 M + H_2O	1 mg de na fase aquosa (liofilizada)
5	300	80 mL de diclorometano	40	2,6 mL de H_2O + 1,4 mL de HCl 0,1 M	1 mg de nisina na fase aquosa (liofilizada)

PCL: Poli(ε-caprolactona). PVA: Poli(álcool vinílico). Mg: miligramas. mL: mililitros. Fonte: a autora (2026).

Ao final da produção das fases, 4 mL da fase aquosa foram pipetados na fase orgânica por ultrassonicação, em gotejamento contínuo, ciclo 1, amplitude 70, por 5 minutos, sendo que o ultrassom permaneceu ligado por 1 minuto e desligado por 30 segundos. Em seguida, o solvente foi evaporado em rotoevaporador. Como controle do teste, as mesmas formulações foram repetidas, sem adição da nisina (Fessi *et al.*, 1989). Ao final, as formulações foram analisadas quanto às suas características físico-químicas e armazenadas em refrigeração, entre 2 e 8 °C.

5.2 DETERMINAÇÃO DO TAMANHO MÉDIO DAS PARTÍCULAS EM SUSPENSÃO E DO ÍNDICE DE POLIDISPERSÃO (PDI):

As suspensões de nanoesferas de nisina e das formulações-controle foram diluídas na proporção 1:100 em H_2O ultrapura e analisadas pelo método de espalhamento dinâmico de luz (DLS) em analisador de partículas Zetasizer Nano (Malvern Instruments©, Malvern, Worcestershire, Reino Unido). As determinações foram realizadas em triplicata e foi determinada a média.

5.3 DETERMINAÇÃO DO POTENCIAL ZETA

As suspensões de nanoesferas de nisina e das formulações-controle foram diluídas nas mesmas condições e analisadas quanto ao potencial zeta em analisador de partículas Zetasizer Nano (Malvern Instruments©, Malvern, Worcestershire, Reino Unido), utilizando ângulo de incidência do laser de 90°. As determinações foram realizadas em triplicata e foi determinada a média.

5.4 RESULTADOS

A Formulação 01 apresentou o melhor desempenho global, com o menor tamanho médio de partícula (467,80 nm) e potencial zeta adequado (-24,40 mV), indicando boa estabilidade coloidal e formação eficiente das nanoestruturas. Entretanto, a proporção relativamente baixa de polímero e estabilizante pode comprometer a estabilidade coloidal a longo prazo, favorecendo agregação progressiva ou separação de fases.

A Formulação 04 destacou-se pelo menor índice de polidispersão (PDI = 0,25), sugerindo maior homogeneidade populacional. Contudo, seu potencial zeta reduzido (-8,81 mV) pode comprometer a estabilidade a longo prazo. A Formulação 03 apresentou o melhor potencial zeta (-27,4 mV), indicando maior estabilidade eletrostática, embora com aumento no tamanho médio de partícula (561,00 nm). Por outro lado, as Formulações 02 e 05 demonstraram desempenho insatisfatório, com valores elevados de tamanho e/ou PDI e baixo potencial zeta, sugerindo maior tendência à agregação e instabilidade coloidal. Em síntese, os resultados obtidos podem ser visualizados na tabela 2.

Tabela 2 – Avaliação do tamanho, PDI e potencial zeta de 5 formulações distinas de nanoesferas contendo nisina.

Formulação	Tamanho (nm)	PDI	Zeta (mV)
01	467,80	0,34	-24,40
02	526,40	0,67	-4,90
03	561,00	0,29	-27,40
04	610,16	0,25	-8,81
05	1146,00	0,53	-9,94

nm: nanômetros. PDI: índice de polidispersão. mV: milivolt. Fonte: a autora (2026).

Tendo em vista que não foi possível desenvolver uma formulação adequada de nanoesferas contendo nisina, optou-se por não seguir os testes microbiológicos em bactérias de otite externa canina e mastite bovina. Desta forma, torna-se necessário delinear novas formulações de nanoesferas contendo nisina, tanto variando os solventes empregados, a concentração de polímero e a forma de incorporação do ativo, mas especialmente o tipo de polímero a ser utilizado.

6 CONSIDERAÇÕES FINAIS

O presente trabalho elucidou o potencial da nisina como alternativa antimicrobiana frente a patógenos de relevância na medicina veterinária. Os resultados demonstram que a nisina possui atividade *in vitro* promissora tanto contra isolados provenientes de mastite bovina quanto de otite externa canina, bem como atividade antibiofilme significativa na inibição da formação e discreta na erradicação do biofilme pré-formado. Adicionalmente, o perfil de citotoxicidade dose-dependente indica que, como aplicação futura, a nisina pode exercer efeito significativo em células procariontes sem danificar células eucariontes, embora sejam necessários estudos que avaliem sua eficácia no contexto da glândula mamária e conduto auditivo.

Além disso, destaca-se a importância de pesquisas futuras testando nanoformulações contendo nisina, capazes de penetrar de forma mais significativa tanto as células sésseis quanto planctônicas e, desse modo, potencializar sua atividade antimicrobiana e antibiofilme. Por fim, o presente trabalho oferece uma contribuição inédita, tendo em vista que após busca realizada não foram encontrados relatos da aplicação da nisina em patógenos da otite canina, reforçando a importância de alternativas aos antibióticos convencionais sob a ótica de Saúde Única.

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