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Characterisation of novel bacteriocins from canine sources

Thesis presented by

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For the degree of

Doctor of Philosophy

University College Cork

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“Winners are not those who never fail, but those who never quit”.

-Edwin Louis Cole

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Date: 28/06/2024 Signed: 

Michelle O'Connor

Contributions

Chapter 2. Creation of nisin immunity clones (*nisFEG* and *nisIFEG*) and resistance clones (*nsr* and *nsrFP*) in pNZ44 was performed by Dr. Miguel Fernández De Ullivarri, Dr. Des Field and Dr. Mariana Perez Ibarreche. Nisin production analysis *via* area under the curve (AUC) assays was performed by Dr. Paula M. O'Connor.

Chapter 3. Mass spectrometry was performed by Dr. Paula M. O'Connor. Creation of nisin ring-B derivative bank was performed by Aoife Granger

Chapter 4. Mass spectrometry was performed by Dr. Paula M. O'Connor. Bioinformatic quality checks, assemblies, and annotations of raw data on whole genome sequenced isolates was performed by Dr. David Hourigan

Chapter 5. Mass spectrometry was performed by Dr. Paula M. O'Connor. Bioinformatic quality checks, assemblies, and annotations of raw data of *Paenibacillus* sp. APC4171 was performed by Dr. David Hourigan. Help with completing phylogenetic analysis of *Paenibacillus polymyxa* genomes was provided by Fabian Thomaz Sebastian Bastiaanssen.

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Publications

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Abbreviations and acronyms

ABC	ATP Binding Casette
AHAW	Animal Health and Welfare
AMR	Antimicrobial resistance
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
ARG	Antibiotic resistance genes
ATP	Adenosine triphosphate
Bce	Bacitracin efflux
BLAST	Basic Local Alignment Search Tool
BHI	Brain Heart Infusion
CAGECAT	CompArative GEne Cluster Analysis Toolbox
CARD	Comprehensive Antibiotic Resistance Database
CD	Crohn's disease
CFS	Cell free supernatant
CHCA	Alpha-cyano-4-hydroxycinnamic acid
CMS	Colony mass spectrometry
Cpr	Cationic antimicrobial peptide resistance
Da	Dalton
DAA	Deferred antagonism assay

Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPG	Diphosphatidylglycerol
EFSA	European Food and Safety Authority
EMBL	European Molecular Biology Laboratory
ESBL	Extended-spectrum beta-lactamase
EU	European Union
FDA	Food and Drug Administration
FT	Freeze thaw
GFP	Green fluorescent protein
GRAS	Generally regarded as safe
GRAVY	Grand average of hydropathicity index
HPRA	Health Product Regulatory Authority
IBD/IBS	Irritable bowel disease/syndrome
IPA	Isopropyl Alcohol
LAB	Lactic Acid Bacteria
LB	Luria Broth
MALDI-TOF	Matrix assisted laser deionised time-of-flight

MIC	Minimum inhibitory concentration
MDR	Multi-drug resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MRS	de Man, Rogosa and Sharpe
MS	Mass spectrometry
MSA	Multiple Sequence Alignment
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NBD	Nucleotide binding domain
NICE	Nisin Controlled Gene Expression system
NCBI	National Centre for Biotechnology Information
OD	Optical density
ONPG	2-Nitrophenyl-β-D-galactopyranoside
PCR	Polymerase chain reaction
PG	Phosphatidylglycerol
pI	Isoelectric point
pLDDT	Predicted local distance difference test
PTM	Post translational modification
RFU	Relative fluorescence units
RiPP	Ribosomally synthesised and post-translationally modified peptides

RLU	Relative light units
rRNA	Ribosomal ribonucleic acid
SIHUMI	Simplified Human Intestinal Microbiota
TCS	Two-component systems
TFA	Trifluoracetic acid
TMD	Transmembrane domain
TOMM	Thiazole/oxazole-modified microcins
TSA	Tryptic Soy Agar
TY	Tryptone, yeast extract
UPP	Undecaprenyl-pyrophosphate
UV	Ultraviolet
VFDB	Virulence Factor Database
VRE	Vancomycin resistant *Enterococcus*
WDA	Well diffusion assay
WGS	Whole genome sequencing
WHO	World Health Organisation

Thesis Abstract

This thesis investigates antimicrobial resistance (AMR) and the potential for bacteriocins as an alternative or adjunct to traditional antibiotics. More specifically, this thesis explores the class Ia bacteriocins termed lantibiotics, and the identification and characterisation of novel bacteriocins from canine sources.

Chapter 1 focuses on the current knowledge regarding the self-protection mechanisms employed by lantibiotic-producing bacteria via their lantibiotic immunity proteins. The research conducted to date on lantibiotic immunity proteins associated with production, as well as the development of lantibiotic resistance in pathogenic species is described, primarily focusing on ABC transporters. This review also discusses potential approaches to overcome lantibiotic resistance.

Chapter 2 investigates the heterologous expression of the nisin immunity proteins NisI and NisFEG, and the nisin resistance proteins Nsr and NsrFP, in three *Lactococcus lactis* strains. One strain is nisin sensitive (MG1614), one is immune (NZ9800), and the third is both nisin producing and immune (NZ9700). This study analysed the sensitivity of these strains harbouring these immunity/resistance proteins using minimum inhibitory concentrations (MIC) assays. This study also determined whether it is possible to increase immunity or resistance to nisin by introducing extra copies of these immunity/resistance genes into strains already exhibiting nisin immunity (NZ9800) or resistance (MG1614 pNP40). The final observation in this study was on the effect of increasing nisin immunity proteins on nisin production in NZ9700.

Chapter 3 investigated bioengineered nisin variants at amino acid residues 9 and 10 within ring-B of the nisin peptide. From this bank of bioengineered derivates a variant, termed nisin M, was found to retain full induction capacity, even at concentrations as high as 300 ng mL^{-1}, while exhibiting a 4-16-fold decrease in antimicrobial activity against nisin sensitive strains.

As the overall aim of this thesis was to investigate the potential for bacteriocins as alternatives or adjuncts to antibiotics, chapter 4 involved screening five canines for novel bacteriocins. A combination of *in silico* and *in vitro* analysis of eight bacterial isolates identified 14 novel bacteriocins, one of which was further characterised. This novel bacteriocin, termed caledonicin, is the first bacteriocin identified from the species *Staphylococcus caledonicus* and exhibits antimicrobial activity against a range of pathogens; including *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus pseudintermedius* (MRSP), and the IBD/IBS associated bacteria *Mediterraneibacter gnavus* ATCC 29149.

The final chapter of this thesis focuses on another bacteriocin identified within the screen described in chapter 4. The lanthipeptide, termed paenidicin L, is a heat stable, protease sensitive peptide which has antimicrobial activity against several indicators. Based on mass spectrometry, it is assumed the peptide consists of five or six (methyl)lanthionine rings.

Overall, this thesis expands the current knowledge on induction and immunity of the most widely studied bacteriocin, nisin, as well as identifies novel bacteriocins from canine sources as potential alternatives to antibiotics in AMR.

Chapter 1. Lantibiotic Immunity and Resistance



Tara

Abstract

Lantibiotics are a distinct class of bacteriocins produced by gram positive bacteria. They are Class I bacteriocins based on their post-translational modifications, heat stability and small size (<5kDa). Lantibiotic operons contain genes required for the production of a mature and active antimicrobial peptide(s), including genes encoding regulation (*lanRK*), maturation (*lanBC* or *lanM)*), leader peptide cleavage (*lanP*) and transport (*lanT*). Lantibiotic-producing bacteria must protect themselves from the action of their own peptide(s) via immunity genes encoding membrane-bound proteins (LanI) and/or ABC transporters (LanFEG). The genes for these immunity mechanisms are also usually located within the bacteriocin operons. However, non-bacteriocin producing bacteria may also be impervious to the action of a bacteriocin due to "resistance" mechanisms which may be specific to the lantibiotic such as the nisin resistance proteins, Nsr and NsrFP, or non-specific resistance mechanisms that bacteria utilise against traditional antibiotics, such as cell wall/membrane modification due to sensory proteins detecting cell wall damage by the action of the lantibiotic. Given that lantibiotics are gaining traction as alternatives to antibiotics in the face of the antimicrobial resistance crisis, it is imperative to understand the mechanisms bacteria use to evade bacteriocin attack. This review will discuss the immunity systems involved in some of the most well-studied lantibiotics, as well as the resistance mechanisms carried by bacteria which protect themselves against these lantibiotics, in particular BceAB-type and Cpr-type ABC transporters. We also discuss the potential to overcome lantibiotic resistance by bioengineering the antimicrobial peptides and combinatorial therapies.

Introduction

Bacteriocins are ribosomally-synthesized, antimicrobial peptides (AMP's) produced by bacteria that target closely related species including known antibiotic-resistant strains (Cotter, Ross and Hill, 2013). A recent review from 2021 by Soltani and co-workers classified bacteriocins into two groups based on the size of the protein (Class I: <5kDa, Class II: 6-10kDa), as well as the presence or absence of modifications within the core peptide. Those with modifications by designated enzymes encoded in the bacteriocin operon are assigned to Class I. Class II undergo no modification, with the exception of potential disulfide bridges to increase stability of the peptide (Soltani *et al.*, 2021). Lanthipeptides belong to the Class Ia bacteriocins and are so named due to the presence of their unusual lanthionine (Lan) and beta methyl-lanthionine (MeLan) ring structures. These rings are enzymatically formed by the dehydration of threonine and serine residues of the peptide, followed by cyclisation by formation of thioether crosslinks between the dehydrated residues and neighbouring cysteine residues (Lubelski, Khusainov and Kuipers, 2009).

Lanthipeptides are further divided into five classes based on the enzymes responsible for their post-translational modifications (PTM). Class I lanthipeptides are modified via dehydration of serine/threonine and subsequent cyclisation by the enzymes LanB and LanC, respectively; Class II are dehydrated and cyclised by one enzyme with an N-terminal dehydration domain and a C-terminal cyclase domain, termed LanM. On the other hand, Class III and IV lanthipeptides are modified via enzymes LanKC and LanL, respectively (Hegemann and Süssmuth, 2020). Lastly, the most recently discovered Class V lanthipeptides have been reported to not contain the typical lanthipeptide modification enzymes in their gene cluster suggesting a non-enzymatic modification, but instead having genes encoding decarboxylase, dehydrogenase, and phosphotransferase activities, as has been suggested for the recently

discovered cacaoidin (Ortiz-López *et al.*, 2020). Lanthipeptides which exhibit antimicrobial activity are referred to as lantibiotics (Arnison *et al.*, 2013).

Lantibiotics are one of the most widely studied classes of RiPPs (ribosomally synthesised and post-translationally modified peptides), with nisin considered the representative bacteriocin of the class (Alkhatib *et al.*, 2012). Bacteriocins, including lantibiotics, have been described as potential alternative treatments to bacterial infections due to their desirable characteristics such as target-specificity, their ability to kill multi-drug resistant pathogens (Cotter, Ross and Hill, 2013; Field *et al*., 2015), and their activity at low concentrations (Meade, Slattery and Garvey, 2020). Another characteristic of bacteriocin-producing bacteria is their capability to tolerate the antimicrobial activity of their own bacteriocin via immunity mechanisms encoded in the bacteriocin operon (or outside). To date the immunity mechanisms for all classes of bacteriocins is still poorly understood (de Freire Bastos, Varella Coelho and da Silva Santos, 2015).

The immunity proteins for lantibiotics are termed LanI (a membrane-bound protein responsible for binding the lantibiotic), and LanFEG (an ATP Binding Cassette (ABC) transporter for exporting the lantibiotic out of the cell membrane and into the extracellular space) (Draper *et al.*, 2008, 2015). These immunity genes have been reported to be conserved to an extent across all lantibiotic producing strains (Alkhatib *et al.*, 2012). However, there have been reports of lantibiotic immunity genes present within non-lantibiotic producing bacteria. One such study investigated the prevalence of lantibiotic immunity/resistance in human gut isolates (Zhang, Cole, *et al.*, 2023). The study analysed 716 bacterial strains and found that 34.5% contained genes encoding LanI and LanFEG proteins within their genomes; in contrast to only 2.5% containing structural and modification genes (*lanA* and *lanBC* or *lanM*). Further investigation of six different metagenomic cohorts spanning five continents made the same observation of

lantibiotic immunity genes being more abundant than structural genes (Zhang, Cole, *et al.*, 2023).

While lantibiotic-producing bacteria protect themselves via these immunity systems there have also been cases reported of non-bacteriocin-producing bacteria displaying resistance to some lantibiotics with similar protein-based protection mechanisms genetically encoded in a biosynthetic gene cluster (Draper *et al.*, 2015).

Here we discuss the protein-based immunity and resistance mechanisms to lantibiotics as opposed to the cell membrane altering mechanisms, although these are briefly mentioned. Due to the limited data available on the immunity and resistance in Class III-V lanthipeptides, we have excluded them from this review. It should be noted this review distinguishes between immunity and resistance mechanisms as follows: immunity is deemed to be the mechanism that allows bacteriocin-producing bacteria the ability to overcome the activity of their own peptide and are normally encoded in the bacteriocin operon. Resistance is used to describe the mechanisms of survival of bacteria against bacteriocins which they do not themselves produce.

Lantibiotic immunity

Nisin Immunity

Nisin is the most widely studied lantibiotic, since its discovery over 100 years ago (Field *et al.*, 2023). Nisin producing strains protect themselves from the action of their own antimicrobial via one or two protein-based immunity systems. These include the LanI membrane-anchored lipoproteins and the ABC transporters LanFEG (Draper *et al.*, 2008), labelled as NisI and NisFEG encoded in nisin operons (Kuipers *et al.*, 1993). All nisin-producing bacteria contain at least one of these immunity mechanisms. Producers of the nisin G, H, S and J variants only express immunity via the NisFEG transporter (O'Connor *et al.*, 2015; O'Sullivan *et al.*, 2020; Lawrence *et al.*, 2022; Sevillano *et al.*, 2023), while all other natural nisin variants have reported both immunity mechanisms encoded in their biosynthetic gene clusters (De Vos *et al.*, 1993; Zendo *et al.*, 2003; Wirawan *et al.*, 2006; De Kwaadsteniet, Ten Doeschate and Dicks, 2008; Hatziioanou *et al.*, 2017; Kim *et al.*, 2019; Garcia-Gutierrez *et al.*, 2020; Sugrue *et al.*, 2023). The lack of the gene encoding the LanI protein within some of these nisin biosynthetic gene clusters is an interesting observation and suggests that these lantibiotic-producing strains no longer require this protection mechanism. However, it has been discovered that the nisin H producer, *Streptococcus hyointestinalis*, does indeed contain a gene encoding a LanI membrane immunity protein, termed *nshI*, co-localised with another membrane transporter gene and response regulator similar to that of the nisin resistance transporter and regulator elsewhere on the genome of the strain, termed *nshFP* and *nshRK* (Ivan Sugrue, personal communication). This revelation suggests perhaps the genes encoding these proteins located outside the nisin H biosynthetic gene cluster may have originated as a result of horizontal gene transfer; or these genes have always been present within the genome, but not identified due to the assumption that genes required for immunity to the bacteriocin should be present within the biosynthetic gene clusters. Indeed, this revelation should warrant further investigation of the genomes of

these nisin variant producing bacteria to confirm that the 'missing' genes are not also located elsewhere on their genomes.

Interestingly, nisin immunity genes have also been found in non-nisin producing strains. One such example is a *L. lactis* dairy isolate, NCDO712, that was found to contain a *nisI* gene located on a novel 50kb plasmid, pNZ712 (Tarazanova *et al.*, 2016). Additionally, Sugrue and coworkers identified a subset of nisin E transporter immunity and regulatory genes (*nseFEGRK*) in 20 *Streptococcus equinus* genomes without finding any evidence of structural or modification genes (Sugrue *et al.*, 2023). They suggest that these 'orphan' immunity genes are widespread across the *S. equinus* species and that resistance to nisin (and/or other antimicrobial peptides) is a considerable factor in the species competitiveness in their natural environments.

Nisin immunity mechanisms include the membrane-anchored lipoprotein, NisI, and the ABC transporter, NisFEG. The NisI pre-protein is a 245 amino acid lipoprotein consisting of two domains (Hacker *et al.*, 2015). Amino acids 16-21 of NisI (GLSGCY) are described as a "lipobox consensus sequence". This sequence acts as a secretion signal for the peptide to be transported out of the cell after the cysteine within this sequence undergoes lipid modification and the first 19 amino acids of the protein are cleaved off (Oscar P. Kuipers *et al.*, 1993; Draper *et al.*, 2008; Jeong and Ha, 2018). This results in a membrane-bound N-terminal anchored to the C-terminal domain via a flexible linker (Khosa, Lagedroste and Smits, 2016). The mode of action of NisI is to bind nisin present in the extracellular environment, thereby preventing the binding of the peptide to lipid II and consequently preventing pore formation (Oscar P. Kuipers *et al.*, 1993; AlKhatib *et al.*, 2014) (Figure 1). While the mode of action of NisI in preventing pore formation specifically is unknown, it is speculated that the C-terminus of NisI can flexibly fold and bind to lipid II, thereby preventing nisin from binding to its target (AlKhatib *et al.*, 2014). This conclusion was as a result of an assay where vancomycin (an antimicrobial that

binds to lipid II without forming pores) was added to nisin sensitive cells, followed by the addition of nisin. No pores were formed in these cells as a result of the lipid II molecules being bound by vancomycin, preventing nisin binding to the same molecule and eventually forming pores (Breukink E *et al.*, 1999). The C-terminus of NisI has been reported to be important in the nisin immunity machinery, where studies conducted on truncated NisI revealed the final 21 amino acids were responsible for providing the majority of nisin immunity, and that in the strain with the truncated protein the immunity was reduced by 86% (Takala and Saris, 2006). Another study used NMR spectroscopy to determine the specific binding site in NisI for nisin and discovered the deletion of these 21 amino acids unravelled the entire C-terminus of NisI, thereby reducing the immunity to almost zero, and the specific binding site in NisI for nisin was determined to be only three amino acids in length (residues 214-216). The same study also reported that the N and C terminal domains of NisI were structurally similar to each other (Hacker *et al.*, 2015) It has also been reported that the binding of NisI to nisin is reversible and that the NisI:nisin complex can dissociate, releasing nisin back into the environment when the antimicrobial levels have dropped (AlKhatib *et al.*, 2014). As well as membrane bound NisI, another study showed that NisI can also be present in a lipid-free form due to the ability of approximately half of NisI escaping lipid modification (Koponen *et al.*, 2004; Jeong and Ha, 2018). This lipid-free NisI acts in the same way as the membrane-bound molecule, in that it reversibly binds nisin within the extracellular environment, but also combines with a second immunity system to create a NisIFEG complex (Koponen *et al.*, 2004; Takala *et al.*, 2004). However, purified lipid-free NisI has been shown to confer nisin protection in a location-specific manner, as exogenously applied lipid-free NisI did not protect cells against nisin (Koponen *et al.*, 2004).

The *nisI* immunity gene is controlled by its own constitutive promoter on the nisin gene cluster, which establishes immunity in the nisin producer before induction by the antimicrobial itself,

while production of the NisFEG proteins is controlled via signal transduction from nisin itself (Li and O'Sullivan, 2006).

The second immunity system involved in protection of producing strains against nisin is NisFEG, a 681 amino acid (aa) complex made up of three proteins (NisF – 225aa; NisE – 242aa; NisG – 214aa). The LanFEG ABC transporter is localized within the cell membrane (Draper *et al.*, 2008). LanFEG acts as an efflux pump exporting nisin molecules that have reached the cell membrane back out into the extracellular environment (Stein *et al.*, 2003) (Figure 1). The NisFEG transporter is made up of NisE and NisG forming the transmembrane domains (TMD) of the transporter, while two NisF proteins possess nucleotide binding domains (NBD) which are located within the cell cytoplasm (Alkhatib *et al.*, 2012). The nucleotide-binding domain of this transporter binds and hydrolyses ATP, which in turn provides energy to the cell to carry out its function of exporting molecules. Deletion of one of the *nisFEG* genes within the nisin operon, and consequent absence of the correlating domain decreases protection and the strain is rendered sensitive to nisin (Siegers and Entian, 1995). The same has been described in the event of NisFEG being capable of binding but not hydrolysing ATP by mutation of the amino acid at position 181 (H_{181}), which corresponds to the conserved sequence motif and essential H-loop region for ABC transporters (Alkhatib *et al.*, 2014). Additionally, despite these immunity proteins being first identified over 30 years ago, their mechanisms are still not fully understood in-particular in the context of co-transcription within the lantibiotic biosynthetic gene cluster, as demonstrated in chapter 2 of this thesis.

In addition to the immunity conferred by the presence of LanI and LanFEG proteins, an increase in nisin immunity was associated with an interruption in the *feuD* gene by transmissible elements in the *L. lactis* N8 nisin Z-producing strain. This genetic mutation affected the membrane potential and therefore binding affinity of nisin to lipid II, and also

changed the composition of the cytoplasmic membrane to enhance nisin immunity (Zhu *et al.*, 2016). Indeed, studies have been conducted where manipulation of the nisin immunity genes for improved nisin production in a nisin-producing strain was performed. These include increased expression of nisin immunity genes *nisI* and *nisFEG* on plasmid-based systems, and the heterologous expression of the nisin response regulatory genes, *nisRK*, on plasmid-based systems (Cheigh *et al.*, 2005; Hu *et al.*, 2010). However, the sensitivity of these strains to nisin was not explored to determine if immunity had been increased. Thus to our knowledge, the ability to improve nisin immunity via increasing the number of copies of immunity genes, and therefore proteins hasn't been demonstrated in nisin producing strains to date.

Immunity to other lantibiotics

Other than nisin other lantibiotic immunity proteins have been identified and studied. Subtilin, produced by *Bacillus subtilus*, is a cationic pore forming lantibiotic with preferential action against gram-positive microorganisms. The subtilin gene cluster is composed of genes for post-translational modification, transport (Klein *et al.*, 1992), regulation (Klein, Kaletta and Entian, 1993), and of course immunity (Klein and Entian, 1994). A previous study uncovered that subtilin immunity and biosynthesis is controlled by a two-component regulatory system SpaRK and the alternative sigma factor H. If the gene encoding sigma factor H is deleted, subtilin biosynthesis and therefore immunity is abolished (Stein *et al.*, 2002). Stein and colleagues elucidated the subtilin immunity machinery by heterologously expressing the genes in the subtilin susceptible *B. subtilis* M01099. Immunity proteins involved in subtilin protection include the lipoprotein SpaI, a protein shown to recognize the C-terminal part of the antimicrobial peptide and thus providing specific immunity against it (Geiger *et al.*, 2019), and the ABC transporter, SpaFEG that is thought to expel subtilin from the membrane to the extracellular matrix (Stein *et al.*, 2005). The *spaE* and *spaG* genes encode proteins that

correspond to the transmembrane domain, while *spaF* and the ATP binding *lanF* share sequence homology, and thus suggests the function of the SpaF protein is the nucleotide binding domain (Stein *et al.*, 2005). Functional analysis of the SpaI protein demonstrated that the C-terminal part of subtilin, but no particular ring structure, is important for SpaI binding. This study also demonstrated that SpaI provided less immunity than SpaFEG but does prevent pore formation more efficiently. It was concluded that SpaI acts as a quick response to protect the cell membrane from subtilin activity (Geiger *et al.*, 2019). Much like the nisin immunity machinery, co-expressing both mechanisms provides the highest level of protection against the peptide (Stein *et al.*, 2005). The structure of the SpaI protein has been described. It is a lipoprotein attached to the outside of the cytoplasmic membrane via a covalent diacylglycerol anchor, where the first 20 N-terminal amino acids of the 15kDa C-terminal domain are required for interaction with the lipid membrane and are required for immunity but are not considered part of the subtilin binding site (Christ *et al.*, 2012). This same study did, however, propose a potential subtilin binding site in a pronounced central hydrophobic surface surrounded by negatives charges. This surface they describe as pointing towards the periplasm of the cell making it a candidate for a binding site for subtilin to SpaI, where it protects the membrane from subtilin insertion (Christ *et al.*, 2012). Interestingly, the nisin immunity protein NisI has been described as having the same fold as the SpaI immunity protein, and the N and C terminal domain of SpaI is structurally similar to the NisI domains - despite a low sequence homology (Hacker *et al.*, 2015).

Another well characterised lantibiotic is lacticin 3147 which is a broad spectrum lantibiotic consisting of two lanthionine containing peptides (LtnA1 and LtnA2) (Morgan *et al.*, 2005). Lacticin 3147 has been shown to exhibit antimicrobial activity via interaction with lipid II by LtnA1 (Wiedemann *et al.*, 2006) and pore formation by insertion of the LtnA2 peptide into the cell membrane (McAuliffe *et al.*, 1998). *L. lactis* subsp. *lactis* (DPC3147) harbours the lacticin

3147 genes on the 60.2kb plasmid pMRC01 (Ma´ *et al.*, 1996). Two divergently transcribed gene clusters on this plasmid are involved in the biosynthesis and immunity of lacticin 3147 (Draper *et al.*, 2008). The cluster encoding immunity to lacticin 3147 is *ltnRIFE* which has been shown to protect it against the lantibiotic (Draper *et al.*, 2008). Interestingly, the expression of these immunity genes (*ltnRIFE*) on a shuttle vector did not confer the same level of immunity observed for the same vector harbouring all genes required for biosynthesis and immunity of lacticin 3147. Further investigation with deletions of *ltnF* and/or *ltnE* genes showed no difference in immunity compared to the vector harbouring *ltnRIFE*; suggesting these genes do not play a role in immunity despite being homologous to ABC transporters. However, deletion of *ltnI* from the vector resulted in complete sensitivity and expression of *ltnI* alone under a strong promoter conferred immunity comparable to wildtype levels. These results overall suggested that lacticin 3147 immunity is only conferred by the LtnI protein (McAuliffe, Hill and Ross, 2000). It is thought that LtnI acts through binding and aggregating lacticin 3147 and therefore blocking its entry into the membrane (Draper *et al.*, 2008). More recent work on the LtnI protein reported amino acids between residues 20 and 27, and 76 and 83 which were essential for its function. Additionally, through mutagenesis and analysis a LtnI protein was found (I81V) that provided enhanced protection. *In silico* analysis of the LtnI protein revealed three hydrophobic transmembrane domains, with an extracellularly located N-terminus and intracellular C-terminus. This same study carried out alignment of the putative amino acid sequences of the LtnI-like proteins identified within other microorganisms and identified conserved regions relating to the putative intracellular loop of LtnI with sharing of mostly charged amino acids across homologues (Draper *et al.*, 2012). A study by Draper and colleagues analysing the cross immunity to lacticin 3147 of a *S. aureus* producer of staphylococcin C55 revealed the potential for lantibiotic resistance through immune mimicry. Genes corresponding to a LtnI-like proteins from *Bacillus licheniformis* DSM 13 and LtnFE-

like proteins from *Lactiplantibacillus plantarum* WCFS1, *S. aureus* ssp. *aureus* NCTC 8325 and *Enterococcus faecium* were expressed from the pNZ44 plasmid. It was found the LtnI-like protein and LtnFE-like proteins from *B. licheniformis* and *E. faecium*, respectively, exhibited levels of resistance just four-fold lower than the immunity provided by the plasmid pMRC01, which contains the entire lacticin 3147 operon. Additionally, a 20-fold greater resistance than that of the sensitive *L. lactis* MG1363 was also observed. This level of self-protection is also just half of that observed for the native *ltnI* gene from the lacticin 3147 producer when expressed on pNZ44, indicating that a relatively high level of protection is provided by these genes (Draper *et al.*, 2009).

Similar to lacticin 3147, the lantibiotic Pep5 (produced by *Staphylococcus epidermidis* 5) is solely reliant on PepI for its immunity (Meyer *et al.*, 1995). Interestingly, Pep5 and epicidin 280 have been demonstrated to exhibit cross immunity (Heidrich *et al.*, 1998). This is in contrast to the lantibiotics subtilin and nisin that share similar structures but do not provide cross immunity (Stein *et al.*, 2003a). This is a phenomenon usually associated with LanI proteins that is suspected to be due to the energy cost on the cell if it was to protect itself from a broad range of AMP's and induce the transcription of an entire lantibiotic gene cluster when a non-self substrate is present (Suárez, Edwards and McBride, 2013). A previous study has shown that PepI functions via protecting Pep5 targets, teichoic or lipoteichoic acids which subsequently prevents pore formation (Hoffmann *et al.*, 2004).

Nukacin ISK-1 is produced by *Staphylococcus warneri* ISK-1, which has 64% identity to lacticin-481 type lantibiotics (Sashihara *et al.*, 2000). NukFEG and NukH have been identified as the proteins to provide immunity to the nukacin ISK-1 producer (Aso *et al.*, 2004). NukH consists of three membrane spanning domains, an internal and external loop. The N terminus of NukH resides inside the cell, while the C-terminus is extracellular. Interestingly, the N- and C-terminal terminus have both been described as not important for immunity, however, amino

acid substitutions in either of the loops abolished immunity function. Additionally, the third transmembrane region is thought to be associated with immunity while the first and second membrane regions are expected to be responsible for lantibiotic binding (Okuda *et al.*, 2005). This same study proposed that NukH inactivates nukacin through binding to the peptide. It has been demonstrated the expression of both nukacin ISK-1 immunity machinery, NukFEG and NukH provide the greatest protection to the producer suggesting a co-operative system in which NukH is an accessory protein to NukFEG (Aso *et al.*, 2005). Mutational analysis in the E-loop domain of the NukF protein revealed the significant role this loop has on the function of the transporter *via* structural changes in the domains. This was determined following replacement of glutamic acid at position 85 to glutamine (E85Q) or alanine (E85A) where a decrease in immunity and transport was observed, but ATPase activity was similar to that of the wildtype transporter (Okuda *et al.*, 2010). It has been reported that residues 1-3 of nukacin ISK-1 are important for NukH binding. This same study also reported that unusual residues such as dehydrated serines and threonines play a role in NukH recognition following mutations at position serine11 and threonine24 of the nukacin peptide and their analysis with peptide binding assays (Okuda *et al.*, 2008).

Lantibiotic resistance

Antimicrobial resistance (AMR) is a growing concern with resistance to current antimicrobials developing at a dangerously rapid pace (Aslam *et al.*, 2018). AMR has existed before the development of even the first antibiotic. Bacterial resistance genes expressing resistance to the first widely used antibiotic, penicillin, were observed in a *Staphylococcus* bacterial strain isolated in 1940, three years before the antibiotics first use as an approved therapeutic in humans in 1943. This is also true for erythromycin, methicillin, vancomycin and gentamicin (Ventola, 2015). Similarly, resistance to bacteriocins has been demonstrated. For example, the first evidence for resistance to nisin was observed in 1984 by McKay and Baldwin (McKay and Baldwin, 1984), over 50 years on from the discovery of nisin in 1928 (Shin *et al.*, 2016). Although bacteriocin resistance is constantly evolving, the rate at which it evolves compared to traditional antibiotics is low (Draper *et al.*, 2015). However, this resistance development and the continuous proposition of bacteriocins as antibiotic alternatives, suggests that resistance to these peptides requires further investigation in terms of mechanistic action, whether these mechanisms are widespread (present in pathogenic or non-pathogenic bacteria), the likelihood of transfer of resistance and methodologies which can be used to overcome this resistance.

Cell membrane modification based resistance

Lantibiotics are cationic in nature and act upon the negatively charged cell membrane (Soltani *et al.*, 2021), therefore it is not surprising that lantibiotic resistance is often due to systems that react to the presence of positively charged peptides or as a response to the cell wall damage inflicted by these peptides (Draper *et al.*, 2015). Such systems which confer resistance to bacteria via manipulation of their cell membrane/wall have been described. These mechanisms do not rely on the production of specific gene proteins but rather involve changes to the bacterial physical or chemical environment.

One such resistance involves alterations to the phospholipid head group in the context of diphosphatidylglycerol (DPG), a negatively charged molecule that can interact with the cationic nisin, allowing it to penetrate deeply into the lipid bilayer. It was found that a nisin-resistant strain of *Listeria monocytogenes* was approximately 12 times more resistant than the wildtype strain following exposure to increasing concentrations of nisin. Analysis of phospholipid headgroup revealed that the nisin resistant strain produced phosphatidylglycerol (PG) instead of DPG and concluded that this reduction in DPG contributed to the enhanced resistance (Verheul *et al.*, 1997).

Biofilms are another well studied phenomenon, especially in the context of lantibiotic resistance. It has been shown that lantibiotics can in fact be effective in preventing biofilm formation, however their ability to kill microorganisms within the biofilm is not as impressive. In a study performed by Okuda *et al*, it was shown that nukacin ISK-1 had no bactericidal activity on biofilms, but it did show bacteriostatic activity against planktonic cells. It was proposed this lack of activity on biofilm cells is due to the mechanism of action of nukacin ISK-1 as lipid II binding but without pore formation (Asaduzzaman *et al.*, 2009), with the hypothesis that pore formation by lantibiotics leads to ATP efflux and is key in eradicating biofilms (Okuda *et al.*, 2013). This same study also tested the activity of nisin and Lacticin Q (a class II bacteriocin) and found these were more efficient at inhibiting both biofilm formation and planktonic cell growth, with nisin A the most efficient. Another study investigating nisin and antibiotic combinations against methicillin resistant *Staphylococcus aureus* (MRSA) biofilms demonstrated the ability for antibiotic-nisin combinations to inhibit biofilm formation but found that the MRSA planktonic cells were not affected by the combinations (Mataraci and Dosler, 2012). For an in-depth discussion on lantibiotics and other bacteriocins targeting biofilms see the review by Mathur and colleagues (Mathur *et al.*, 2018).

Lastly, another characterised mechanism of resistance is the potassium (K^+) efflux system/ion channel. For example, mutants of *L. monocytogenes* strains F6861 were isolated with increased nisin resistance. An ELISA (Enzyme-linked immunosorbent assay) was performed to analyse the adsorption of nisin to these strains. It was shown that the amount of nisin that adsorbed to the cell reflected the sensitivity of the strain to the antimicrobial, where sensitive cells showed a greater adsorption to nisin. In contrast, resistant strains demonstrated an increase in the potassium efflux system with increasing nisin concentrations, indicating a reduction in suitable sites for nisin binding (Davies and Adams, 1994).

Protein-based resistance mechanisms

Interestingly, despite the fact that many lantibiotics target the peptidoglycan precursor lipid II such as nisin (Breukink E *et al.*, 1999), lacticin 3147 (Wiedemann *et al.*, 2006) and nukacin ISK-1 (Asaduzzaman *et al.*, 2009), it has been suggested the levels of this precursor molecule is not involved in resistance mechanisms involving cell wall modification (Draper *et al.*, 2015). There are reviews which discuss in detail the cell membrane altering resistance mechanisms, some of which are briefly mentioned above (reader is directed to reviews by de Freire Bastos, Varella Coelho and da Silva Santos, 2015; Draper *et al.*, 2015).

As previously mentioned, in addition to these cell wall/membrane modification resistance mechanisms there is also protein-based resistance conferred by the presence of proteases or ABC transporters in the cell membrane. Currently there are two types of ABC transporters that confer resistance to lantibiotics, the cationic antimicrobial peptide resistance (CprABC) type transporters, which bear resemblance to the lantibiotic LanFEG (Field *et al.*, 2023); or the bacitracin efflux ATP binding (BceAB) type transporters (Clemens *et al.*, 2018). The current knowledge on the mechanisms of resistance to lantibiotics, in particular nisin, via these protein

resistance mechanisms and possible interventions to overcome this are discussed below.

Nisin resistance proteins Nsr and NsrFP

While nisin has been described as a promising alternative to antibiotics, there have been reported cases of nisin resistance in pathogenic bacteria including *Streptococcus agalactiae* (Khosa, AlKhatib and Smits, 2013; Hayes *et al.*, 2019), *L. monocytogenes* (Collins *et al.*, 2010) and *Staphylococcus aureus* (Blake, Randall and O'Neill, 2011; Randall *et al.*, 2018).

Nisin resistance mechanisms include the nisin resistance protein, Nsr (Sun *et al.*, 2009). Nsr is a serine protease that cleaves nisin between the modified cysteine amino acid at position 28, and serine positioned at 29 in the N-terminal region of nisin (Sun *et al.*, 2009; Field *et al.*, 2019). The three-dimensional structure of the Nsr protein has been solved recently, and it consists of an eleven alpha-helices and eleven beta-strands to form a total of three domains, the N-terminal helical domain, the protease cap domain and the core protease domain, the latter which contains the active site of the protease known as the TASSAEM site. The protease cap domain is proposed to form a lid like structure above the tunnel (Khosa *et al.*, 2016) which contains the active TASSAEM site where Ser236 is the catalytically active residue (Khosa, AlKhatib and Smits, 2013). This TASSAEM site has been proposed to form a catalytic dyad for nisin cleavage by Nsr, *via* interaction with the highly conserved His98 residue located at the end of the N-terminal helical bundle (Khosa *et al.*, 2016). Additionally, modelling of the Nsr protein interacting with the nisin peptide suggested hydrophobic residues of Nsr protease core domain (Leu102, Leu137, Met173, Ile174, Ala277) and the polar residues Asn172 and Glu266 bind to rings D and E of nisin. Further analysis of this model revealed that three of these residues (Asn172, Met173 and Ile174) form a pocket that harbours rings D and E (Khosa *et al.*, 2016).

While most proteases are incapable of degrading the nisin protein due to the presence of

lanthionine rings causing steric hindrance, Nsr differs in that it recognises and binds the C-terminal ring-E of nisin at one end of the proteases catalytic tunnel and guides nisin through the core domain (Khosa *et al.*, 2016) (Figure 1). *Nsr* was first found to be plasmid encoded on the 40 mega-dalton plasmid pNP40 (McKay and Baldwin, 1984), however the gene can also be found within a nisin resistance operon together with genes encoding a two-component regulatory system, termed NsrRK, and a second resistance mechanism, the ABC transporter nsrFP (Khosa, AlKhatib and Smits, 2013).

Indeed, while cheese starters may include *L. lactis* strains that produce nisin and therefore are immune to the antimicrobial peptide, there have also been several reports of the production of Nsr in *L. lactis.* In fact, as already noted above, *nsr* was first found to be plasmid encoded in a *L. lactis* strain DRC3 (then known as *Streptococcus lactis*) (McKay and Baldwin, 1984). However, another study found that 38% of 270 strains of *L. lactis* analysed carry the *nsr* gene (Lieke A. van Gijtenbeek *et al.*, 2021). Other studies have found similar findings, for example *nsr* was found to be plasmid located in 10% of *L. lactis* strains isolated from fresh milk samples (Yun, Jing and Chen, 2006). A screen of 179 milk samples identified *L. lactis* TS1640 that had nisin resistance via a plasmid encoded *nsr* gene (Tang *et al.*, 2001). Another study identified *nsr* located on a 56kb plasmid in *L. lactis* (Liu, Harvey and Dunn, 1997). This widescale presence of *nsr* on large plasmids suggests this resistance is as a result of horizontal gene transfer.

Additionally, as mentioned previously the *S. hyointestinalis* nisin H producer was found to encode a *nsrFP* like transporter (termed *nshFP*) in its genome (Ivan Sugrue, personal communication). This finding highlights the ability for nisin resistance mechanisms to transfer to other bacterial genera or species, regardless of their lantibiotic producing potential.

The second protein based nisin resistance mechanism is NsrFP, an ABC transporter which, like

NisFEG, is driven *via* ATP hydrolysis. NsrFP belongs to the Bce class of transporters that are poorly understood (Furtmann *et al.*, 2020a). The mechanism of action of the NsrFP resistance transporter was poorly understood until recently (Gottstein *et al.*, 2022).

NsrP consists of 212 amino acids acting as the transmembrane domains of the resistance protein, while NsrF is the nucleotide binding domain responsible for ATP hydrolysis (Reiners *et al.*, 2017). NsrP is predicted to consist of 10 transmembrane helices, unlike NisE and NisG with six transmembrane helices each (Siegers and Entian, 1995; Reiners *et al.*, 2017). The NsrF protein was recently characterised *via* comparative modelling, as the nucleotide binding domains are described as the most conserved region of ABC transporters. It was found the histidine residue at position 202 (H_{202}) was conserved within the H-loop domain of the protein, and when a mutation was made ($H_{202}A$) and expressed heterologously the strain no longer exhibited nisin resistance. This lack of resistance was found to be due to the mutant NsrFP being incapable of hydrolysing ATP. This study also compared all nucleotide triphosphates to determine the most favourable to NsrF and confirmed ATP had the highest affinity to the protein and therefore is the preferred co-factor (Furtmann *et al.*, 2020).

While the C-terminus of nisin has been determined to be important for interacting with the nisin immunity transporter NisFEG (Alkhatib *et al.*, 2014), it is the N-terminus of the lantibiotic which is important for NsrFP recognition and exporting the peptide out of the cell (Reiners *et al.*, 2017) (Figure 1). A recent study by Gottstein and co-workers revealed new insights into this resistance protein, whereby heterologous expression of NsrFP in *L. lactis* protected the cells to several AMP's including nisin, bacitracin and lysobactin, an interesting observation as these compounds are structurally different from each other. They also reported an accumulation of lipid II cell wall precursor molecules in the cytoplasm of sensitive cells treated with bacitracin, an expected result considering the AMP's mode of action is binding the pyrophosphate moiety undecaprenyl-pyrophosphate (UPP) in cells. However, cells expressing

the ABC transporter did not have this accumulation, concluding NsrFP prevented bacitracin from binding UPP. Interestingly, proteomic cell-based assays showed increased production of cell wall modification enzymes such as RodA and MurQ, both involved in cell wall synthesis in the NsrFP expressing *L. lactis* strain compared to the sensitive control. Additionally, an ATP deficient NsrFP mutant demonstrated the ATP dependency of this transporter. These results from Reiners (2017) and Gottstein (2022) demonstrate that NsrFP confers resistance *via* multiple modes of action including export of the antimicrobial into the extracellular environment, increasing the production of cell wall modification enzymes, and the protection of the target UPP, by releasing it from the grip of the antimicrobials (Reiners *et al.*, 2017; Gottstein *et al.*, 2022).

Resistance to other lantibiotics

The development of lantibiotic resistance is not limited to nisin. Other miscellaneous resistance mechanisms in pathogenic bacteria have been described, some of which are discussed below.

Another BceAB transporter has been well researched and described, the BceAB transporter in *Bacillus subtilus.* This was the first BceAB transporter reported as conferring bacitracin resistance (Bernard *et al.*, 2003; Ohki *et al.*, 2003). The BceAB system has been shown to be activated *via* the BceRS two component system as a result of direct interaction with bacitracin (Ohki *et al.*, 2003). However, it has since been reported that the transmembrane protein, BceB, and the sensory kinase BceS interact directly, independent of bacitracin presence, demonstrating communication between the TCS and transporter for resistance (Dintner *et al.*, 2014). It has also been shown the presence of BceAB transporter is required for activation and transcription of the *bceA* promoter unveiling a unique induction system (Bernard *et al.*, 2007). The mode of action of the transporter via binding of BceB and bacitracin for translocation

across the membrane has also been proposed (Dintner *et al.*, 2014). In contrast to this study, a mechanism of resistance has been reported in which the concentration of drug-target complexes drives transporter activity where lipid II is freed from the grip of the antimicrobial (Kobras *et al.*, 2020). This ABC transporter has since been reported in other bacterial species including *Enterococcus faecalis* (Manson *et al.*, 2004) and *Streptococcus mutans* (Tsuda *et al.*, 2002) and confers resistance to the lantibiotics mersacidin and actagardine (Staroń, Finkeisen and Mascher, 2011). This same study also described the PsdAB transporters of *B. subtilus* that confer resistance primarily to lipid II binding lantibiotics like nisin and gallidermin, but also confers resistance to subtilin and actagardine (Staroń, Finkeisen and Mascher, 2011).

In addition to BceAB type transporters, another type of transporter has been reported. This is the CprABC type transporter. This transporter was first described by McBride and Sonenshein (2011) following isolation of *Clostridioides difficile* mutants on media containing nisin which exhibited resistance up to three times the inhibitory concentration of the peptide compared to sensitive cells (McBride and Sonenshein, 2011). This study demonstrated that the CprABC transporter conferred resistance to nisin, Gallidermin and polymyxin B (McBride and Sonenshein, 2011). The gene cluster of this resistance transporter is comprised of *cprABC*, where *cprA* corresponds to the NBD and *cprBC* encode the transmembrane domains of the proteins of the transporter protein. The remainder of genes within the operon are the response regulator and histidine kinase *cprR* and *cprK*, respectively which have been shown to control and activate the CprABC system (Suárez, Edwards and McBride, 2013; Clemens *et al.*, 2018). It has been shown that these Cpr regulators are activated by a variety of lantibiotics produced by a diverse assortment of Gram-positive organisms. The authors concluded that the CprABC transporter and its regulators are replicas of lantibiotic immunity systems that evolved to recognize multiple substrates and defend *C. difficile* against these antimicrobial peptides made by other bacteria in the intestine (Suárez, Edwards and McBride, 2013). It has been suggested

the rings A and B of structurally similar lantibiotics (nisin, subtilin, gallidermin and mutacin 1140) are an important motif for *cpr* recognition and activation (Suárez, Edwards and McBride, 2013). Another study conducted by Ide and colleagues evaluated the susceptibility of eleven *C. difficile* isolates, which contained this Cpr transporter, to nisin A and found that CprA expression in all strains was induced by nisin A. They also found amino acid differences in the sequences of CprA, CprB, and CprC amongst the strains which exhibited differing levels of nisin resistance, speculating that these differences are related to nisin susceptibility. Interestingly, these differences in susceptibility were also observed for lantibiotics epidermin and mutacin III, both of which are structurally similar to nisin (Ide *et al.*, 2023). In conclusion, this ABC transporter is of concern as it has been demonstrated to confer resistance to several lantibiotics (McBride and Sonenshein, 2011; Suárez, Edwards and McBride, 2013; Ide *et al.*, 2023). Differences between the BceAB-type and CprABC-type transporters involved in lantibiotic resistance have been reviewed by Clemens and colleagues (Clemens *et al.*, 2018).

Briefly, other ABC transporters responsible for lantibiotic resistance include the BraDE and VraDE transporters, both of which have been found to be activated via the BraRS two-component system. These systems confer resistance to nisin and bacitracin (a polypeptide antibiotic) in *S. aureus* strains (Hiron *et al.*, 2011). The co-expression of a third protein, the VraH transmembrane protein with VraDE also confers high level of resistance to gallidermin (Popella *et al.*, 2016). Two transporter systems, VirAB and AnrAB, confer resistance to nisin, bacitracin, and other antimicrobials with differing modes of action in *L. monocytogenes* (Collins *et al.*, 2010). This resistance mechanism is harboured by the food-associated pathogen *L. monocytogenes* EGD-e and is thought to be non-specific given the many antimicrobials the transporter system targets (Jiang *et al.*, 2019). It was also reported that the VirAB transporter acts as a sensor molecule for the VirSR two component system, whereby deletion of *virAB* rendered a sensitive strain to cefotaxime and the sensory kinase VirS no longer sensed the

presence of the antimicrobial without VirAB (Jiang *et al.*, 2019). This was further suspected to be due to the presence of an extracellular loop domain in the VirB permease thought to be essential in antimicrobial signalling. The mandatory presence of the transporter for sensing antimicrobials is unknown but has been the subject of some speculation. One such explanation is the proteins transports the antimicrobial directly to the sensory kinase or there is communication between VirRS and the transporter as has been described for BceB and BceS in *B. subtilus*, described above (Jiang *et al.*, 2019).

Overcoming lantibiotic resistance

While the development of resistance to lantibiotics in an AMR crisis is cause for concern, as mentioned previously this rate of resistance is much lower than resistance to traditional antibiotics (Draper *et al.*, 2015) Fortunately, advances in next generation sequencing (NGS) and genome assembly and the availability of large genome datasets allows for quicker and broader analysis to monitor the development of resistance to lantibiotics (Zhang, Wu, *et al.*, 2023). In addition, genome mining tools and artificial intelligence (AI) databases will be of significant use in monitoring bacteriocin resistance development, including lantibiotic resistance, within microbial niches. AI can also help to predict genetic modifications of existing peptides for the "best fit" to overcome these resistance mechanisms (Field *et al.*, 2023). These tools will also aid in studies which aim to discover novel lantibiotic variants (Tietz *et al.*, 2017; Van Heel *et al.*, 2018; Blin *et al.*, 2023; Abramson *et al.*, 2024), as demonstrated previously where 8,500 lanthipeptide precursor peptides were identified across 100,000 genomes analysed (Walker *et al.*, 2020). This analysis is one example of the use of these tools in the identification of lantibiotics within large metagenomic datasets. These *in silico* approaches will aid tackle lantibiotic resistance earlier than that of resistance development to traditional antibiotics (Ventola, 2015).

Additionally, a beneficial characteristic of bacteriocin and lantibiotic producing bacteria is their genetically encoded nature, allowing for genetic engineering of the pro-peptide structural gene and therefore bioengineering of the antimicrobial peptide to produce variants with enhanced characteristics, for example enhanced antimicrobial activity (Chen *et al.*, 2013; Hayes *et al.*, 2019; Twomey *et al.*, 2020), enhanced specific activity (Field *et al.*, 2021) including targeting Gram negatives (Yuan *et al.*, 2004; Field, Begley, O'Connor, *et al.*, 2012), enhanced solubility (Yuan *et al.*, 2004) or variants which can overcome resistance mechanisms identified within pathogenic bacteria to aid in the AMR crisis (Field *et al.*, 2019).

Two pathways which can be employed to overcome lantibiotic resistance are discussed below. These include bioengineering of lantibiotics for enhanced properties against pathogens, and combinatorial therapies which have demonstrated promising results for use against MDR pathogens.

Lantibiotic bioengineering

As mentioned previously, lantibiotics can be subjected to bioengineering to produce variants with enhanced characteristics. While numerous bioengineering studies have been carried out on nisin, there are limited studies investigating nisin variants specifically with an aim to overcome the resistance mechanisms discussed in this review.

One such study that focused on overcoming the nisin resistance protein, Nsr was conducted by Field and colleagues (2019) where substitutions of a serine to a proline at position 29 (S29P) and an isoleucine to a valine at position 30 (I30V) rendered a Nsr-resistant nisin derivative (Field *et al.*, 2019). This Nsr-resistant derivative, designated nisin PV, demonstrated enhanced antimicrobial activity against 64.8% of 122 *Streptococcus agalactiae* strains, where 120 of these were found to confer nisin resistance *via* Nsr. This study demonstrated the ability of the bioengineered derivative to overcome the resistance in these strains (Hayes *et al.*, 2019).

Another study compared the activity of nisin A and nisin H, including a bioengineered nisin H variant, F1I (Reiners *et al.*, 2020). This study carried out IC_{50} assays with these nisin variants against *L. lactis* NZ9000 strains harbouring *nis*I, *nisFEG*, *nsr* and *nsrFP* genes on a plasmid and observed almost all strains displayed a reduction in protection when treated with the nisin H F1I peptide in comparison to nisin A and nisin H.

Similar to the study by Reiners and colleagues, another study analysed the nisin A peptide at position Ile1 by mutating this amino acid to all other possible residues. They compared the

nisin Ile1X derivatives antimicrobial activity against sensitive strains and strains expressing the nisin resistance proteins Nsr and NsrFP, and the immunity proteins NisI and NisFEG. Substitution of isoleucine with aromatic residues resulted in variants with enhanced activity against all four proteins. One particular mutant, I1W, showed high potency against NisI, NisFEG and Nsr with a higher IC_{50} value when targeting NsrFP but still lower than that observed for the wildtype peptide. They ranked the I1W and I1F mutants as exhibiting greater activity than wildtype with I1Y exhibiting less activity compared to wildtype. This study also demonstrated the decreased activity for variants involving substitution with charged amino acids and residues capable of undergoing modification by the NisB/NisC enzymes (S or T) (Lagedroste *et al.*, 2019a). Additionally, another study conducted by this same group looked at bioengineered nisin hinge region variants, including elongating this region and observed their activity against *L. lactis* NZ9000 harbouring the *nisI, nisFEG*, *nsr* and *nsrFP* genes. One derivative with two additional amino acids (IV) in the hinge regions ($_{20}$NMKIV$_{24}$) exhibited enhanced activity against the strains expressing the NisFEG and NsrFP proteins. Here, it was observed both strains expressing these proteins were almost two-fold more sensitive to this nisin variant compared to wildtype (Zaschke-Kriesche *et al.*, 2019).

As mentioned earlier, rings A and B have been suggested to be an important motif for Cpr recognition and activation in lantibiotic resistant *C. difficile* (Suárez, Edwards and McBride, 2013). Based on this observation and assumption, mutational analysis of ring A and B of lantibiotics nisin, mutacin 1140, gallidermin and subtilin could be an option for overcoming this resistance. However, based on the requirement of rings A, B and C for nisin induction, biosynthesis of its own peptide and the contribution of these ring structures to the lantibiotics antimicrobial activity (Rink *et al.*, 2007), mutation of these ring structures in lantibiotics could prove to be difficult.

Other work currently being performed by our group suggests that mutations to the most C-terminally located ring (ring E) of nisin suggests the importance of this location in relation to the function of the nisin resistance and immunity mechanisms, NisI, NisFEG, Nsr and NsrFP (described above) (Des Field, personal communication, unpublished).

As previously stated, the rate at which lantibiotic resistance is developing is much lower than that reported for traditional antibiotic resistance (Draper *et al.*, 2015). However, based on the relatively minute scale of use of these peptides in human and animal settings compared to traditional antibiotics it is likely that the level of lantibiotic resistance would increase further, should bacteriocins and lantibiotics be widely used in clinical settings. This likely outcome suggests that these peptides should be investigated further to future-proof their usefulness and overcome any future lantibiotic resistance development. As suggested by Cotter and colleagues, if bioengineering of lantibiotics is to be employed for specific uses, including overcoming lantibiotic resistance "*an appreciation of the roles of each individual amino acid (and each domain) is required*" (Cotter *et al.*, 2006). Indeed, this approach will depend on the length of the peptide, however two studies which took this approach have demonstrated the residues which are essential for antimicrobial activity in lacticin 3147 (Cotter *et al.*, 2006) and nukacin ISK-1 (Islam *et al.*, 2009). While many studies have been conducted on bioengineering lantibiotics for overcoming antibiotic resistant bacteria and indeed these bioengineered derivatives could be targeting these pathogens *via* overcoming their resistance mechanisms, including lantibiotic resistance mechanisms, the specific mode of action of these peptides have not been elucidated. Perhaps future lantibiotic engineering should focus on testing these derivatives on bacteria which heterologously express the specific lantibiotic resistance mechanisms to test the ability of these derivatives to overcome these this issue, as has been demonstrated by Reiners and co (Reiners *et al.*, 2020), Lagedroste and co (Lagedroste *et al.*, 2019a) and Zasche-Kriesche and co (Zaschke-Kriesche *et al.*, 2019).

Combinatorial therapies

Another approach to combat lantibiotic resistance is to apply these peptides in combination with other membrane targeting peptides or antibiotics. This has already been suggested and demonstrated for lantibiotics and antibiotic combinations against MDR pathogens (Field *et al*., 2015). For example, it was attempted to fuse the N-terminal tail of peptides that target gram-negative bacteria to the entire nisin peptide or to create a nisin variant containing all five rings followed by an anti-gram-negative tail. The aim of this study was to create a nisin hybrid molecule which was capable of passing through the outer membrane of gram-negative organisms. One variant (T16m2) displayed four to twelve times more activity than wildtype nisin against all gram-negative indicators tested (Li, Montalban-Lopez and Kuipers, 2018). A similar study looked at the binding of vancomycin with the lipid II binding fragment of nisin (residues 1-12) with the hypothesis that the combination of two lipid II binding moieties in one molecule which bind separate parts of the target (nisin binding UPP and vancomycin binding the pentapeptide) could restore the activity against vancomycin resistant enterococci (VRE). Here one particular compound, designated compound 5 in the study, was highlighted for exhibiting 40 times more activity than the nisin and vancomycin fragments separately. This compound interestingly also exhibited activity against the gram-negative *Klebsiella*, where vancomycin alone was reported to show no activity (Rishi *et al*., 2014).

Combinations of lacticin 3147 and polymyxin B and E exhibited synergistic activity against gram-negative pathogens, in particular against *Cronobacter* and *Escherichia coli.* This study demonstrated this combination provides a mean for expanding the targets of the lantibiotic while also reducing the concentration of polymyxin as a result of this synergy between the antimicrobials (Draper *et al.*, 2013).

Nisin when used in combination with cefotaxime or ceftriaxone, both β-lactam antibiotics, demonstrated synergy and enhanced activity against clinical isolates of *Salmonella enterica*

serovar Typhi highlighting the possibility of using these combinations while newer formulations are being developed (Rishi *et al.*, 2014).

The combination therapies mentioned above do not address specific lantibiotic resistance mechanisms, similar to the lantibiotic bioengineering strategies discussed earlier. However, these studies do pave the way for combination therapies to be a potential option for overcoming the lantibiotic resistance mechanisms which have been described considering their potent activity against MDR pathogens.

Conclusion

Many lantibiotic resistance transporters have been identified and discussed in detail in recent years. One review by Draper and colleagues discusses lantibiotic resistance mechanisms in detail, including cell membrane modifications. Here, we briefly discussed some of the resistance mechanisms involved in cell membrane modification but with a particular focus on the most characterised protein-based resistance mechanisms. This is principally the case for the nisin resistance protein Nsr and the transporter NsrFP, which have more recently been described in terms of function and structure; as well as other well-researched ABC transporters involved in lantibiotic resistance.

Nisin is the most studied of lantibiotics and perhaps of all bacteriocins with decades of research following its discovery 100 years ago (Field *et al.*, 2023). This, in combination with nisin being the representative lantibiotic amongst this bacteriocin class, means that the majority of lantibiotic immunity studies focus primarily on this lantibiotic, as is evident in this review. Although the wealth of research on the nisin peptide and the genetic elements within the biosynthetic operon over the last century paves the way for a better understanding of how their corresponding proteins work, the mechanism of action of the immunity proteins have not been fully elucidated. This is particularly the case for research conducted on the manipulation of these systems for enhanced desirable effects on lantibiotic biosynthesis, such as increased lantibiotic yield, where it has been suggested increasing the number of immunity proteins in lantibiotic producing bacteria could increase production of the lantibiotic itself (Cheigh *et al.*, 2005; Ni *et al.*, 2017). This is discussed in greater detail in chapter 2 of this thesis.

While bacteriocins have been described as potential alternatives to antibiotics in the AMR era (Cotter, Ross and Hill, 2013) there have also been cases of bacteriocin resistance reported, some of which are summarised in this review. While these resistance mechanisms are not yet completely understood in comparison to the resistance mechanisms to traditional antibiotics

that are currently on the market, it is clear based on the apparent lack of research as noted here, that more studies need to be conducted specifically to bioengineer lantibiotics with the ultimate aim to overcome these lantibiotic resistance mechanisms if they are to fulfil their promise as the next generation of antimicrobials in the fight against AMR .

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Figures

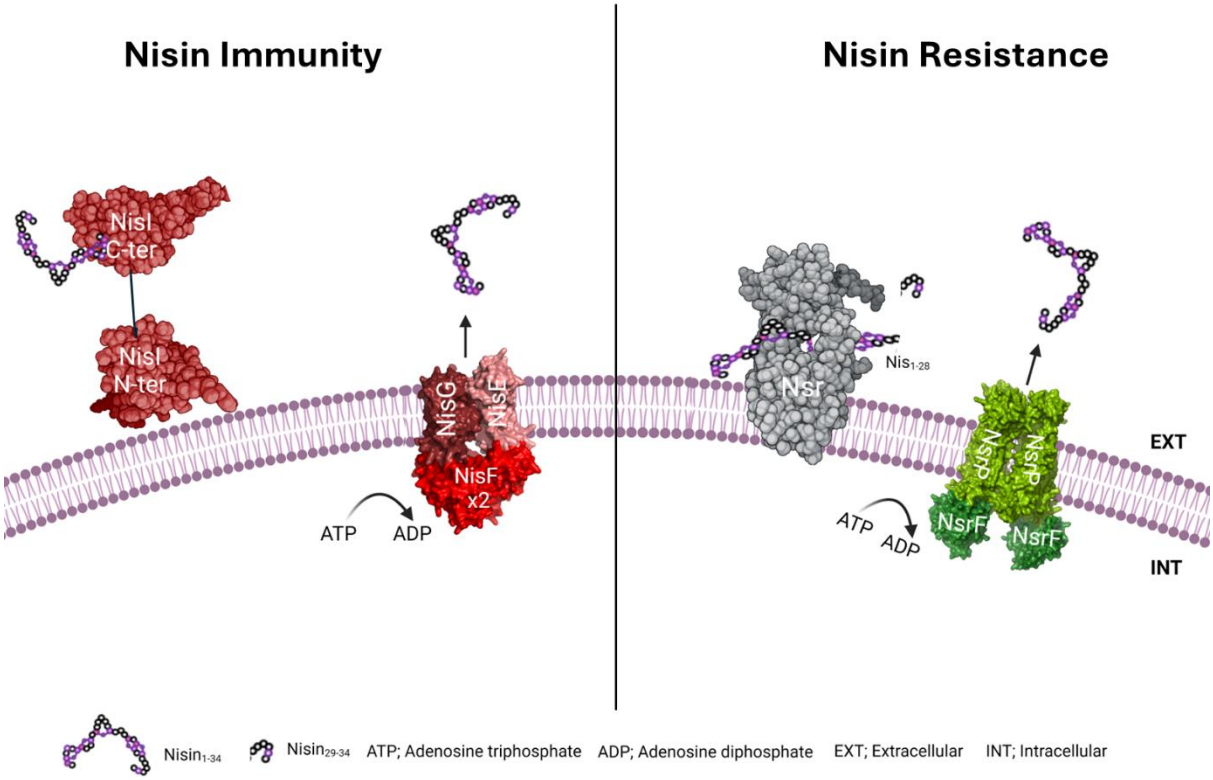


Figure 1. Schematic overview of nisin immunity and resistance mechanisms NisI, NisFEG, Nsr and NsrFP. The membrane bound N-terminus of the NisI protein is bound to the C-terminus via a flexible linker. The C-terminus of NisI binds to the N-terminus of the nisin peptide, preventing the peptide from binding to its target molecule, lipid II. The nucleotide binding domain (NBD) NisF is activated following phosphorylation. NisFEG recognises the C-terminus of nisin and guides the peptide into the extracellular environment. Nsr recognises and binds ring-E of nisin and guides the peptide through the catalytic tunnel which contains the active TASSAEM site. NsrF is also activated upon phosphorylation and recognises the N-terminus of nisin for extracellular exportation.

NisI and Nsr proteins accessed through RCSB Protein Databank (PDB) (Accession numbers: NisI N-terminus; 2N32, NisI C-terminus; 2N2E, Nsr; 4Y68). N- and C-terminal domains of NisI are attached via a flexible linker. NisFEG and NsrFP proteins were folded on Alphafold3 (https://alphafoldserver.com/) where a predicted template modeling (pTM) score of 0.78 and 0.5 was

observed, respectively, indicating the overall predicted fold for the complexes might be similar to the true structure. Subsequent visualisation of the NisFEG and NsrFP models was carried out in PyMOL Molecular Graphics System, v 3.0 (Schrödinger, LLC). (Image created with BioRender.com)

Chapter 2. Relative contributions of nisin immunity and resistance mechanisms to protection of *Lactococcus lactis* from nisin.



Ollie

Abstract

Over the last number of decades, antimicrobial peptides have become a topic of interest for use in conjunction with, or as alternatives to traditional antibiotics, due to their ability to target clinically-relevant pathogens. One such class of these antimicrobial peptides are bacteriocins and in particular, the lantibiotics. While the most studied characteristic of lantibiotics relates to their potent inhibitory activity, there are other distinguishing features associated with these peptides that warrant further exploration. These include the ability of the bacteriocin producer to protect itself from the action of its own peptide via self-immunity. In particular, nisin-producing bacteria protect themselves *via* dedicated proteins termed NisFEG and NisI. This study aimed to expand the current knowledge on the levels of protection conferred by the nisin immunity (NisI, NisFEG) and resistance systems (Nsr and NsrFP) by introducing their genes in *Lactococcus lactis* strains MG1614 (nisin sensitive), NZ9800 (nisin immune) and NZ9700 (nisin immune and nisin producing). The genes were cloned in a high-copy plasmid, pNZ44, under the control of the strong P44 constitutive promoter. We determined the consequences of overexpression of these genes on strain growth as well as nisin production in *L. lactis* NZ9700. We found that while NisI and NisFEG confer the same level of protection when expressed alone, the two protein systems provide the greatest immunity when expressed together, with a >120X increase in immunity compared to the control. However, *L. lactis* MG1614, or the *nis*A knockout strain NZ9800, harbouring vectors providing increased copies of immunity genes could not replicate the degree of immunity exhibited by the natural nisin-producing strain NZ9700. This suggests that the nisin immunity conferred by naturally producing strains is optimised to overcome the antimicrobial peptide, with up to a 960X increase in immunity over sensitive controls. We further demonstrate that increased immunity in the nisin-producing strain NZ9700 does not impact levels of nisin production.

Introduction

Bacteriocins are ribosomally-synthesized, antimicrobial peptides (AMPs) produced by bacteria that usually target closely related species, including known antibiotic-resistant strains (Cotter, Ross and Hill, 2013). These AMPs can be grouped into two fundamental classes based on the presence (Class 1) or absence (Class 2) of post-translational modifications (Cotter, Hill and R. Paul Ross, 2005; Walsh *et al.*, 2021). Class I contains the bacteriocins termed lanthipeptides, characterised by the presence of lanthionine (Lan) and methyl-lanthionine (MeLan) ring structures. Lanthipeptides that exhibit antimicrobial activity are termed lantibiotics (Arnison *et al.*, 2013).

Nisin is a 34 amino acid lantibiotic produced by a variety of bacterial genera and species, including *Lactococcus lactis* (a number of variants, nisin A, Q, Z, F, are produced by lactococci) (De Vos *et al.*, 1993; Zendo *et al.*, 2003; De Kwaadsteniet, Ten Doeschate and Dicks, 2008). Other nisin variants are produced by *Streptococcus uberis*, *hyointestinalis*, *equinis*, *agalactiae*, and *salivarius* (nisin U and nisin U2, nisin H, nisin E, nisin P and nisin G, respectively) (Wirawan *et al.*, 2006; O'Connor *et al.*, 2015; Garcia-Gutierrez *et al.*, 2020; Lawrence *et al.*, 2022; Sugrue *et al.*, 2023b), *Staphylococcus capitis* (nisin J) (O'Sullivan *et al.*, 2020), *Ligilactobacillus salivarius* (nisin S) (Sevillano *et al.*, 2023) and *Blautia obeum* (nisin O) (Hatziioanou *et al.*, 2017). Nisin exhibits a broad spectrum of activity, targeting numerous gram-positive pathogens including a number of human pathogenic species including *Staphylococcus*, *Listeria*, *Clostridia* and *Bacillus* (Field *et al.*, 2015). Its mode of action primarily involves the targeting of the cell wall peptidoglycan precursor, lipid II (Breukink *et al.*, 2003), following which it forms pores within the cell membrane, consequently leading to leakage of cell constituents (Wiedemann *et al.*, 2001). Nisin displays reduced activity against gram-negative bacteria due to the presence of an outer membrane protecting the lipid II-containing inner membrane (Li, Montalban-Lopez and Kuipers, 2018).

An essential characteristic of bacteriocin-producing bacteria is their ability to protect themselves from the action of their own bacteriocin *via* immunity mechanisms. This is due to the action of a membrane-bound lipoprotein, LanI, and a multi-subunit ATP Binding Cassette (ABC) transporter, LanFEG (Draper *et al.*, 2008). Some nisin producers are protected by the presence of a single immunity mechanism (LanI), as reported for the natural nisin variants, nisin H (O'Connor *et al*., 2015) nisin J (O'Sullivan *et al*., 2020), nisin G (Lawrence *et al*., 2022), and nisin S (Sevillano *et al*., 2023). But most nisin-producers possess two immunity mechanisms (LanI and LanFEG), this is the situation for all other nisin variants discovered to date (Sugrue *et al*., 2023). The lipoprotein, NisI is composed of two domains (Hacker *et al*., 2015). The protein contains a consensus sequence which allows the protein to undergo lipid modification (Oscar P. Kuipers *et al.*, 1993; Jeong and Ha, 2018), resulting in a 226 amino acid membrane-bound protein with the N-terminal domain anchored to the C-terminus via a flexible linker (Khosa, Lagedroste and Smits, 2016). It is thought that NisI acts by binding to nisin, thereby preventing formation of a nisin-lipid II complex and consequent pore formation (Oscar P. Kuipers *et al.*, 1993; AlKhatib *et al.*, 2014).

The second immunity system harboured by all nisin producers is the LanFEG-type ABC transporter (belonging to the ABC-2 subfamily of multidrug resistant (MDR) proteins (Lubelski, Konings and Driessen, 2007) localized within the cell membrane (Draper *et al.*, 2008). NisFEG consists of two integral transmembrane domains, NisE and NisG, and two NisF nucleotide-binding domains in the cell cytoplasm which are responsible for providing energy to the protein complex *via* ATP hydrolysis (Takala *et al.*, 2004; Alkhatib *et al.*, 2012). The function of NisFEG is to act as an efflux pump exporting nisin molecules from inside the cell membrane into the extracellular environment (Stein *et al.*, 2003). The two systems have been described as working co-operatively to offer maximum protection to a nisin-producing strain (Alkhatib *et al.*, 2014).

While nisin has been described as a promising alternative to antibiotics due to its potent antimicrobial action, there have been reports of nisin resistance development in pathogenic bacteria (Collins *et al.*, 2010; Blake, Randall and O'Neill, 2011; Khosa, AlKhatib and Smits, 2013; Hayes *et al.*, 2019). For clarity, we characterise those systems associated with nisin production and encoded within the nisin operon as immunity mechanisms, whereas for those not linked to production, we term resistance mechanisms. To date, two specific protein-based nisin resistance mechanisms have been reported, including the serine protease, Nsr (Sun *et al.*, 2009), and the bacitracin efflux (Bce) type ABC transporter, NsrFP.

The Nsr protein is a serine protease first found to be encoded on the 65kb plasmid pNP40 in *L. lactis* DRC3 (McKay and Baldwin, 1984). However, *nsr* can also be found within a larger nisin resistance operon that includes a two-component system, termed NsrRK, and also in combination with genes encoding NsrFP (Khosa, AlKhatib and Smits, 2013). Nsr is reported to cleave nisin between ring E and serine 29 (Field *et al.*, 2019), resulting in a significantly less bioactive nisin peptide. The BceAB-type transporter, NsrFP, acts similarly to NisFEG in that it exports nisin out of the cell. The complex consists of a transmembrane protein NsrP and a nucleotide binding protein, NsrF (Reiners *et al.*, 2017).

A schematic representation of the nisin immunity (NisI and NisFEG) and nisin resistance (Nsr and NsrFP) mechanisms are presented in Figure 1.

Here we aim to dissect the contribution of nisin immunity and resistance mechanisms to non-nisin-producing and nisin-producing strains *via* heterologous expression of *nisI*, *nisFEG*, *nsr* and *nsrFP* and evaluate the sensitivity of these strains to nisin. We also examined the effect of introducing these genes on strain growth and determined whether or not increasing nisin immunity had an effect on nisin production in a nisin-producing background.

Materials and Methods

Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. Strains of *L. lactis* were grown in M17 broth/agar supplemented with 0.5% glucose (GM17) at 30 °C. *Escherichia coli* Top10 cells were grown in Luria-Bertani (LB) broth/agar at 37°C. Antibiotics were used where indicated at the following concentrations: Chloramphenicol (Cm) at 10 µg mL^{-1} for both *L. lactis* and *E. coli* strains harbouring the pNZ44 plasmid.

Molecular cloning and transformation of nisin immunity/resistance genes

All coding sequences used for cloning were amplified by PCR with Phusion DNA Polymerase (Thermo Fisher, Ireland) and cloned into the *E. coli/L. lactis* shuttle vector pNZ44, under control of the strong constitutive P44 lactococcal promoter. *nsrFP* was amplified from *Streptococcus agalactiae (S. agalactiae*) COH1 gDNA (Genbank accession: GCA_000689235.1), sequences of *nisI* and *nisFEG* were amplified from *Lactococcus lactis* (*L. lactis*) NZ9700 gDNA (Genbank accession: HM219853.1), and those of *nsr* were amplified from the nisin resistance encoding plasmid, pNP40 (Genbank accession: M37002.1). *nsrFP*, *nsr*, *nisFEG* and *nisIFEG* were cloned by the restriction/ligation method using T4 DNA ligase according to the manufacturer's indications (Thermo Fisher), while *nisI* was cloned with the InFusion® HD Cloning Kit (Takara) as per manufacturer's instructions with primers first designed to amplify the gene downstream of a ribosomal binding site (RBS) (NisI-RBSFor & NisIRev); and subsequent amplification with primers containing overlapping regions for the pNZ44 plasmid and restriction sites for NcoI and HindII [pNZ44NisIFor(NcoI) & NisIpNZ44Rev(HindIII)]. This second set of primers was designed using the online Takara primer design tool (https://www.takarabio.com/learning-centers/cloning/primer-design-and-

other-tools). *NsrFP* and *nisFEG* were directionally cloned using the NcoI/PstI and NcoI/HindIII restrictions sites, respectively. To clone the *nisIFEG* genes, *nisI* was non-directionally cloned into pNZ44-*nisFEG* using the HindIII restriction site. Ligation reactions were used to transform chemically competent *E. coli* TOP10 and transformants were selected in LB agar plates supplemented with 8 µg mL^{-1} chloramphenicol. Transformants were screened by colony PCR with primers pNZ44-For/Rev and sequences of the cloned vectors were confirmed by DNA sanger sequencing. Recombinant plasmids were propagated and purified from *E. coli* TOP10 cultures and transformed by electroporation into electro-competent *L. lactis* MG1614, NZ9800 and NZ9700 strains. Primers used for molecular cloning are summarized in Table 2.

Minimum inhibitory concentration assays

Minimum inhibitory concentration (MIC) determinations were performed in triplicate in 96-well microtitre plates (Sarstedt). The 96-well microtitre plates were pre-treated with bovine serum albumin (BSA) prior to addition of the peptides. Briefly, to each well of the microtitre plate, 200 µL of phosphate-buffered saline (PBS) containing 1% (w/v) BSA (PBS/BSA) was added and incubated at 37 °C for 30 min. The wells were washed with 200 µL PBS and allowed to dry. Target strains were grown overnight in the appropriate conditions and medium as described above, sub-cultured into fresh broth and allowed to grow to an OD600 of ~0.5. Cells were diluted to a final concentration of 10^5 cfu mL^{-1} in a volume of 0.2 mL. Wild-type nisin A was adjusted to a 0.5 µM, 15 µM or 30 µM starting concentration for MG1614, NZ9800 or NZ9700 strains, respectively. Two-fold serial dilutions of peptide were added to the target strain. After incubation for 16 h at 30°C, the MIC was read as the lowest peptide concentration causing inhibition of visible growth. MICs were carried out in triplicate for each target strain.

Following the analysis of MIC results of the NZ9800 control, induction of the strain was carried out with pure nisin M peptide. Briefly, at the sub-culture stage of MIC assays, NZ9800 was induced with 50 ng mL $^{-1}$ of pure nisin M peptide purified as previously described (O'Connor *et al.*, 2020). The remainder of the MIC assay was carried out as described above.

Growth curve assays of nisin immunity/resistance expressing *L. lactis* strains

For growth experiments, fresh overnight cultures of *L. lactis* MG1614, NZ9800 and NZ9700 test strains and controls were grown as described above for MIC assays and cells were adjusted to 10^5 cfu mL^{-1}. To each well of the 96-well microtitre plate, 200 µL aliquots of each strain were added. Cells were grown statically at 30°C and measured spectrophotometrically at an OD600 over 24-h periods at 1 h intervals (with shaking for 5 s before each reading) using a Multiskan FC spectrophotometer (Thermoscientific). Experiments were performed in triplicate.

Assessment of nisin bioactivity *via* deferred antagonism assay and well diffusion assays

Deferred antagonism assay (DAA)

Nisin production analysis of NZ9700 strains with increased immunity (see Table 6) was analysed via three methods. Briefly, 10 µL of nisin-producing strains harbouring immunity determinants (alone or in combination) and appropriate controls were spotted on M17 agar plates supplemented with 0.5% glucose (GM17) and incubated overnight at 30 °C. Following UV irradiation of spotted cultures, deferred antagonism assays were performed with indicator strain *L. lactis* HP and incubated overnight at 30 °C.

Well diffusion assay (WDA)

Well diffusion assays (WDAs) were also performed with cell-free supernatant (CFS) from these strains against *L. lactis* HP. Briefly, following the spotting of strains on GM17 agar for DAAs, strains were subjected to centrifugation (4500 rpm for 10 min) and passed through a filter (0.2 µm) to obtain CFS. WDAs were performed by boring wells into GM17 agar previously seeded with a 1% inoculum of *L. lactis* HP, and 50 µl of CFS was added to wells. Zones of inhibition for both DAAs and WDAs were measured using Vernier callipers (resolution 0.05), recorded in millimetres (mm) and rounded to one decimal place. Activity was calculated as follows: diameter of zone − diameter of colony/well in millimetres and expressed as the average between two. Results for both assays were plotted as column tables and statistical analysis was performed using Graphpad Prism v 8.0.1. All assays were performed in triplicate.

Nisin quantification via area under the curve (AUC)

Reversed-phase HPLC analysis was used to quantify nisin A concentrations in CFS of the nisin-producing strains *L. lactis* NZ9700 (with and without extra immunity determinants, Table 6) by comparing the area of the nisin A peak for each strain to a nisin A standard curve.

Generation of nisin A standard curve

To generate the nisin A standard curve, a 1000 µM nisin A stock solution was diluted 1 in 100 with Milli Q water to give a starting concentration of 10 µM. This was then serially diluted 1 in 2 to give 5, 2.5 and 1.25 µM solutions of nisin A. One hundred µL aliquots of each standard dilution were run on an analytical Aeris C18 reversed-phase HPLC column (4.6 x 10 mm, 5µ, 100Å, Phenomenex, Cheshire, UK) running a 27-37% gradient at 1 mL min^{-1} over 20 minutes where mobile phase A is Milli Q water containing 0.1% TFA and

mobile phase B is 100% acetonitrile containing 0.1% TFA. The nisin A peak area was plotted for each concentration to generate a standard curve which was used to quantify the nisin A concentration in the CFS.

Preparation of strains for analysis

Twenty-five µl of each strain (from stock) was inoculated into 5 mL GM17 broth and incubated overnight at 30 °C. Twenty-five µL of the GM17 overnight was then used to inoculate 5 ml TY broth and again incubated overnight at 30°C. One mL of each overnight culture was centrifuged and the CFS retained for nisin quantification. One hundred µL aliquots of CFS from strains of interest were applied to the HPLC as described above and the nisin A peak area was compared to the nisin A standard curve to quantify the amount of nisin (µM) produced by each strain. Results are reported in table format and also plotted in GraphPad Prism v 8.0.1 where statistical analysis was performed. This assay was performed in duplicate.

Statistical analysis

Statistical analysis for all growth curves and nisin quantification assays in this study were performed in GraphPad Prism v8.0.1.

For growth curve assays, each strain's replicate values were fitted with a non-linear curve of regression to obtain the YM, Y0, k and Xint values, where YM and Y0 are the maximum population/carrying capacity and starting population, respectively, both reported in OD_{595}; k is the rate constant reported as OD_{595}/time (hours); and Xint, also known as 1/k, is the length of lag phase reported as time (hours). Subsequent tests of normality were performed on these separate values for all strains within the growth curves. Where normality was assumed, a one-

way ANOVA was performed to determine if differences were present in three phases of growth in the test strains when compared to the controls in each growth curve. The three phases of growth statistically analysed were lag phase (Xint), growth rate (k) and carrying capacity (YM), in comparison to the controls in each growth curve. For ANOVA results with a significant difference between groups (P <0.05) a post hoc test was performed. The post-hoc test for normally distributed/equal variances assumed samples was the Dunnet test. The significance threshold for all ANOVA tests performed was set at 0.05.

For analysis of nisin quantification, tests of normality were performed for all assays. Strains displaying normally distributed data were further analysed with a one-way ANOVA where all strains were compared against each other within the test for nisin quantification. The post-hoc test performed for nisin quantification was Tukey's multiple comparison test. The significance threshold for all ANOVA tests performed was set at 0.05.

Results

Effect of nisin immunity and resistance proteins on nisin sensitivity

All MIC assays in this study were performed using HPLC-purified nisin A from *L. lactis* NZ9700 (Table 1). The MIC was determined to be the lowest concentration of peptide which resulted in the absence of visible growth of the target strain after 16 hours under the appropriate growth conditions. It is important to note the strains MG1614 (Nis^-, Imm^-), NZ9700 (nisin-producer; Nis^+, Imm^+) and NZ9800 (NZ9700 with a 4 bp deletion in *nisA*; Nis^-, Imm^+) are isogenic in that all are derivatives of MG1363 (Table 1).

Initial MIC assays were performed on *L. lactis* MG1614 (Nis^-, Imm^-) harbouring one or a combination of the nisin immunity and resistance determinants (*nisI*, *nisFEG*, *nisIFEG*, *nsr* and *nsrFP*) to determine the degree of protection provided by each system to nisin. *L. lactis* MG1614, NZ9800 and NZ9700 were used as controls for comparison to each strain under investigation. The protein systems that offer the most protection from nisin are NisIFEG and NsrFP when compared to the MG1614 control, where a 120 fold increase in protection was observed for both (MIC of 1.875 µM). This level of self-protection decreased to 60 fold for NisI and NisFEG separately (MIC of 0.938µM each) and to 30X for Nsr (MIC of 0.468µM). Interestingly, NsrFP offers the same level of protection as NisIFEG, despite the presence of a single transporter as compared to the dual immunity systems provided by NisIFEG. Introduction of pNZ44*nisIFEG* into *L. lactis* MG1614 provided the same level of immunity as NZ9800. The NZ9800 strain does not produce nisin and thus would not be expected to induce its own NisFEG system (under control of P*nisF*), whereas pNZ44nis*IFEG* is continuously expressed under the constitutive promoter p44. Notably, no strain under investigation in this study was capable of providing the equivalent level of protection that was observed for the nisin producer NZ9700, that harbours a single chromosomal copy of *nisI* and *nisFEG* and provides a 960-fold increased tolerance compared to the MG1614

control (MIC of 15µM) (Table 3). This tolerance of NZ9700 is likely to be even higher than our calculation since the strain is capable of producing its own nisin in addition to the exogenously added nisin in our assay.

Further MICs were performed to determine the effect of introducing extra copies of the nisin resistance determinants *nsr* and/or *nsrFP* into an MG1614 strain which already contains the natural plasmid, pNP40 (Nsr+). The results revealed that increasing the copy number of the *nsr* gene under p44 provided greater nisin resistance in that this strain was twice as resistant (MIC of 0.468µM) as a strain containing pNP40 alone (MIC of 0.234µM). Surprisingly, although pNZ44*nsrFP* confers a high degree of resistance (MIC of 1.875µM) compared to strains harbouring pNP40, pNZ44*nsr* or both in combination (Nsr^{++}), the introduction of pNZ44*nsrFP* into MG1614 harbouring pNP40 (Nsr^{+}, $NsrFP^{+}$) brought about a reduction in the MIC value to that of MG1614 pNP40 (0.234µM). This was unexpected given that the genes encoding Nsr, NsrFP and a cognate two-component response regulator NsrRK are found clustered in several pathogenic strains as an operon that resembles the genetic architecture of the *nisI* and *nisFEG* immunity genes found in nisin-producing strains (Khosa, AlKhatib and Smits, 2013).

In addition to investigating the effects of *nsr* and *nsrFP* overexpression in terms of resistance to nisin, the effects of increasing copies of the nisin immunity genes, *nisI*, *nisFEG* and *nisIFEG* in the NZ9800 (Nis^{-}, Imm^{+}) strain was also investigated. The presence of additional copies of *nisI* and *nisFEG* resulted in a two-fold increase in the level of protection (MIC of 3.75µM) in comparison to the NZ9800 control (MIC of 1.875 µM). Moreover, introduction of the pNZ44*nisIFEG* construct increased the immunity by a factor of four (MIC of 7.5µM). Despite this expected increase in immunity, it still did not reach the level exhibited by the nisin-producing strain NZ9700 (MIC of 15µM (equivalent to 50ug mL^{-1} nisin)). This finding

suggests the genome-situated nisin biosynthetic operon is somehow optimised for the survival of the strain, a phenomenon that is still not fully understood (Table 5).

Unexpectedly, the NZ9800 (Nis^-, Imm^+) strain harbouring the empty pNZ44 vector, exhibited a 4-fold decrease in MIC(0.468µM) in comparison to the control. Notably, this increased sensitivity to nisin was not apparent with MG1614 (Nis^-, Imm^-) (Table 3) or NZ9700 (Nis^+, Imm^+) (Table 6) strains harbouring the empty vector.

Given that NZ9700 and NZ9800 only differ by their ability to produce nisin, and that the nisin biosynthetic cluster is auto-regulated by the peptide, we examined the sensitivity of NZ9800 following exposure to exogenously applied nisin at a relatively low cell density (10^5 cfu mL^{-1}) prior to carrying out the MIC assay. To that end, induction of the NZ9800 strain was carried out with nisin M, a bioengineered nisin A variant with significantly decreased antimicrobial activity but with the same induction capacity as the wild-type peptide (O'Connor *et al.*, 2020). 50 ng mL^{-1} of nisin M was utilised which would not significantly contribute to the antimicrobial activity of the nisin A peptide used in our MIC assay but would still induce transcription of *nisIFEG via* the two-component system, NisRK. However, this induction step did not appear to increase the MIC of NZ9800 thereby establishing that pre-induction of the nisin operon in NZ9800 does not increase immunity to the levels of the nisin producer, NZ9700 (Table 5).

Finally, the impact of introducing extra copy numbers of nisin immunity genes in NZ9700 (Nis^+, Imm^+) and any subsequent impact on nisin production (discussed later) was investigated. Notably, the increased copy numbers of *nisI* effectively doubled the immunity of the strain (MIC of 30 µM) when compared to the control (MIC of 15µM) (Table 6). In contrast, increased copies of *nisFEG* resulted in a decrease in immunity to half that of the control (MIC of 7.5µM). Meanwhile, when all four genes were expressed together on the

pNZ44 vector (*nisIFEG*) an MIC of 15µM was observed which was equivalent to that of the NZ9700 control strain (Table 6).

Effect of nisin immunity and resistance mechanisms on bacterial growth

Growth curves were employed to assess whether or not the presence of increased copies of the immunity genes affected strain growth and were carried out concurrently with MIC assays with all bacterial cells diluted to the equivalent concentration used in MICs (10^5 cfu mL^{-1}). Statistical analysis (ANOVA) was performed by comparing the test strains to the control strain (i.e. strain without plasmid pNZ44) in each assay and involved comparing the lag phase (Xint), the growth rate (k) and carrying capacity (YM) of all strains to the control.

Growth curves were employed to assess the impact on nisin-sensitive MG1614 harbouring all pNZ44 constructs (Figure 2), the impact of introducing the nisin resistance determinants (pNZ44*nsr*, pNZ44*nsrFP*) in a strain already harbouring a resistance mechanism (in this case Nsr on the plasmid pNP40) (Figure 3), or increasing copies of nisin immunity genes *nisI*, *nisFEG* and *nisIFEG* in a strain already harbouring nisin immunity which does not produce nisin (NZ9800 Nis^-, Imm^+) and in a strain harbouring nisin immunity which does produce nisin (NZ9700 Nis^+, Imm^+) (Figure 4 and 5).

Growth curves of MG1614 strains harbouring all pNZ44 constructs (pNZ44, pNZ44*nisI*, pNZ44*nisFEG*, pNZ44*nisIFEG*, pNZ44*nsr* and pNZ44*nsrFP*) demonstrated no statistically significant difference for carrying capacity for most of the strains, with the exception of MG1614 carrying pNZ44*nsr* or pNZ44*nsrFP* ($p < .05$). Growth rate analysis (k) showed no significant difference amongst all strains in this assay. The length of the lag phase demonstrated a significant difference between the control and test strains containing plasmids pNZ44 ($p < .05$), pNZ44*nisI* ($p < .005$), pNZ44*nsr* and pNZ44*nsrFP* ($p < .0001$) (Figure 2).

Carrying capacity, growth rate and length of lag phase of MG1614 strains harbouring the pNP40 plasmid and either pNZ44*nsr* or pNZ44*nsrFP* were also calculated (Figure 3). The maximum population attained by these strains was significantly different between MG1614 containing pNZ44*nsr* and pNZ44*nsrFP* in comparison to the control, MG1614, where the strains harbouring the nisin resistance determinants on pNZ44 (pNZ44*nsr* or pNZ44*nsrFP*) had decreased carrying capacity ($p < 0.05$). There was no significant difference in growth rate across all strains in comparison to the MG1614 control in this assay. However, a negative effect on growth was also observed for the carrying capacity and length of lag phase for MG1614 pNZ44*nsrFP* when compared to MG1614 with and without the pNP40 plasmid. However, the growth rate of this strain and all strains in this assay displayed no significant difference to the control. Results observed for lag phase analysis showed significant differences between all test strains when compared to MG1614 (MG1614 pNZ44 $p < 0.005$; MG1614 pNP40 + pNZ44 and MG1614 pNP40 + pNZ44*nsrFP* $p < 0.0005$; MG1614 pNZ44*nsr* and MG1614 pNZ44*nsrFP* $p < 0.0001$), with the exception of the strain harbouring pNP40, and strain harbouring pNP40 + pNZ44*nsr* (Figure 3).

Growth curves of NZ9800 (Nis^-, Imm^+) with and without pNZ44 with nisin immunity genes (pNZ44,pNZ44*nisI*, pNZ44*nisFEG* and pNZ44*nisIFEG*) demonstrated no significant difference in carrying capacity for all strains in comparison to the control, NZ9800 (Figure 4). However, comparison of growth rate showed a significant difference between NZ9800 and the strain harbouring pNZ44*nisI* ($p < 0.05$). Unexpectedly, the only strain exhibiting a statistically significant difference in length of lag phase to the control was N9800 pNZ44*nisFEG* ($p <.05$), where a shorter lag time was observed before exponential growth began after 4 hours compared to 6 hours for the control. This result suggests the extra copies of the ABC transporter caused a significant decrease in lag time, an interesting finding given

that when copies of the NisI immunity protein, or both proteins (NisIFEG) on pNZ44 are present in this strain there was no difference when compared to the control (Figure 4).

Growth curves of NZ9700 ($Nis^{+,}$ Imm^{+}) demonstrated a significant difference in the maximum levels for NZ9700 carrying pNZ44, pNZ44*nisI* and pNZ44*nisFEG* when compared to the control ($p < 0.05$), but no difference for NZ9700 with construct pNZ44*nisIFEG* (Figure 5). This was in contrast to the NZ9800 constructs mentioned above, where no differences in max population were observed (Figure 4). No significant difference was observed between the growth rate of all strains compared to the control, indicating that the generation time for nisin-producing strains is not affected by the presence of increased immunity proteins. Lag phase of growth was affected only for the NZ9700 strain carrying pNZ44*nisI* when compared to the strain without the plasmid ($p < 0.05$).

Effect of increased immunity on nisin production

Deferred antagonism and well diffusion assays

The most widely used method for the quantification or detection of nisin bioactivity is the DAA. When DAAs were employed, a significant difference between the control strain NZ9700, and test strains NZ9700 pNZ44*nisFEG* and NZ9700 pNZ44*nisIFEG* was evident by a significant decrease in nisin production in terms of inhibitory zone size ($p < 0.0001$ and $p < 0.05$, respectively). All other test strains compared to the control revealed no significant difference (Figure 6A). When NZ9700 pNZ44*nisFEG* and NZ9700 pNZ44*nisFEG* pre-treated (PT) with nisin M were assessed no significant difference in bioactivity was observed (Figure 6A). This is likely due to the nature of the assay which involves colonies of cells in close proximity to one another and supporting more efficient induction by the *nisRK* two-component system.

However, when CFS from the NZ9700 and those harbouring pNZ44 with immunity genes were analysed, there was no significant difference across all strains compared to the control, except for the CFS from NZ9700 pNZ44*nisFEG* ($p < 0.005$), which displayed no antimicrobial activity (Figure 6B). As observed previously with DAAs, there was no significant difference between the NZ9700 pNZ44*nisFEG* induced and un-induced strains (Figure 6A). In the case of NZ9700 pNZ44*nisFEG* growing in liquid media, it is likely that the increased copies of the NisFEG transporter resulted in the nisin inducer being exported from the cell in the early stages of production, resulting in too low a concentration for further induction via the two-component regulatory system, NisRK. This hypothesis was supported by the significant difference observed amongst CFS tested for the uninduced NZ9700 pNZ44*nisFEG* and induced with nisin M at 50 ng mL^{-1} ($p < 0.05$) (Figure 6B).

Area Under the Curve Analysis (AUC)

AUC analysis was employed on NZ9700 and pNZ44, pNZ44*nisI*, pNZ44*nisFEG*, and pNZ44*nisIFEG* transformants to determine the nisin concentration based on a standard curve of nisin A concentrations (1.25, 2.5, 5 and 10 µM) (Figure 7). Nisin concentration determinations of test strains were only compared to the control strain, NZ9700.

Although initial observations would suggest a small increase in nisin production in the NZ9700 strain harbouring the empty vector (pNZ44) when compared to the control NZ9700 based on the AUC concentrations calculated, it became apparent that there was no difference in terms of nisin production following statistical analysis (Figure 7C). The only statistically significant difference observed was for NZ9700 harbouring constructs pNZ44*nisFEG* and pNZ44*nisIFEG* ($p < 0.005$) (Figure 7C). Here, an average nisin concentration of 0 µM and 0.49µM, respectively (Figure 7B) compared to 1.59 µM for the wild-type producer was

observed, which is also represented visually via WDAs of these samples in Figure 6A. Deferred antagonism (Figure 6A), well-diffusion assays (Figure 6B) and AUC analysis (Figure 7A-C) of all NZ9700 strains in this study reveal that providing increased copy numbers of immunity genes, *nisI*, *nisFEG* and *nisIFEG* in trans and under the control of a strong, constitutive promoter, does not positively impact nisin production in the NZ9700 strain.

Discussion

While antimicrobial resistance has become an ever-growing topic within AMP research, there is a paucity of studies investigating the role and impact of nisin immunity mechanisms and specific nisin resistance mechanisms that counteract the antimicrobial action of nisin, in stark contrast to the enormous number of studies conducted pertaining to its antimicrobial activity. Nisin, for example, is one of the most widely studied bacteriocins since its discovery in the early 20th century, in particular for its broad spectrum of activity (Field *et al.*, 2023). Nisin-producing bacteria protect themselves from the action of their own antimicrobial via one (nisin G, H, J & S) or multiple protection mechanisms (O'Connor *et al.,* 2015; O'Sullivan *et al.,* 2020; Lawrence *et al.,* 2022; Sevillano *et al.,* 2023). With the aim to further add to the knowledge surrounding nisin immunity and resistance, we investigated a number of these protein-based protection mechanisms (NisI, NisFEG, NisI/FEG, Nsr and NsrFP), to determine the contribution of each of these mechanisms to provide protection from nisin. This was accomplished via heterologous expression of the nisin immunity/resistance genes outlined above into a nisin-sensitive strain of *Lactococcus lactis* (MG1614), and comparing the protection conferred when expressed separately or in combination with one another. Additionally, this study aimed to determine whether nisin immunity/resistance can be further augmented by providing extra copies of immunity/resistance genes to an already nisin immune (*L. lactis* NZ9800) or nisin resistant (*L. lactis* MG1614 pNP40) strain and examining the resultant effect on the growth of the strains. The final objective of this study was to examine the impact of extra copies of nisin immunity genes on nisin production in strains via AUC analysis in comparison to controls.

Nisin immunity

Nisin immunity is conferred via four proteins, NisI, NisF, NisE and NisG, the last three of which form the ABC transporter NisFEG. NisI is a lipoprotein consisting of an N and C-

terminal domain (Hacker *et al.*, 2015). NisI contains what is described as a "lipobox consensus sequence" which acts as a secretion signal for the protein to be transported out of the cell after undergoing lipid modification (Oscar P. Kuipers *et al.*, 1993; Jeong and Ha, 2018), resulting in a membrane-bound N-terminal, anchored to the C-terminal domain via a flexible linker (Khosa, Lagedroste and Smits, 2016). The C-terminus of NisI has been reported to be important in nisin immunity, and studies have shown that the final 21 amino acids are responsible for providing the majority of nisin immunity as a deletion mutant resulted in an 86% reduction in immunity (Takala and Saris, 2006). Another study conducted by Hacker and co-workers (2015) determined the specific binding site in NisI for nisin at amino acid positions 214-216 (Hacker *et al.*, 2015). The binding of NisI to nisin is reversible and the NisI:nisin complex dissociates when environmental nisin levels are low (AlKhatib *et al.*, 2014). *nisI* is under the control of an independent constitutive promoter, P*nisI*, which provides the cell with low levels of immunity (Li and O'Sullivan, 2006). This weak promoter acts as a preliminary defense mechanism for initial interaction with the peptide, before full immunity is expressed through the activation of the two-component regulatory system, NisRK, and subsequent production of NisFEG (Draper *et al.*, 2008).

The NisFEG transporter is made up of two transmembrane proteins (TMD), NisE and NisG and a nucleotide binding protein (NBD) NisF, present at a ratio of 2:1:1 for NisF, NisE and NisG, respectively (Takala *et al.*, 2004; Alkhatib *et al.*, 2012). NisF binds and hydrolyses ATP, which provides the energy to export nisin through NisE and NisG. Deletion of one of the three genes (*nisF/E/G*) within the nisin operon, and subsequently deletion of the correlating domain renders decreased protection and renders the producing strain increasingly sensitive to nisin (Siegers and Entian, 1995).

Nisin resistance

Nisin resistance has been reported in several bacterial species including in clinically relevant bacteria such as *Streptococcus agalactiae* (Khosa, AlKhatib and Smits, 2013; Hayes *et al.*, 2019), *Listeria monocytogenes* (Collins *et al.*, 2010) and *Staphylococcus aureus* (Blake, Randall and O'Neill, 2011; Randall *et al.*, 2018). Protein-based nisin resistance mechanisms characterised to date include the nisin resistance protein, Nsr (Sun *et al.*, 2009) that cleaves nisin between residues 28 and 29 producing a peptide with significantly decreased antimicrobial activity (Sun *et al.*, 2009; Field *et al.*, 2019).

Another protein-based nisin resistance mechanism is NsrFP, a BceAB-type ABC transporter (Furtmann *et al.*, 2020b). In contrast to NisFEG however, the mode of action of NsrFP is poorly understood (Gottstein *et al.*, 2022). NsrP acts as the transmembrane protein, while NsrF is the nucleotide-binding protein responsible for ATP hydrolysis (Reiners *et al.*, 2017). NsrFP was recently found to confer resistance via multiple modes of action and to multiple antimicrobial peptides, including bacitracin and nisin (Gottstein *et al.*, 2022). This was an interesting observation as these compounds are structurally different from each other. Studies have shown that NsrFP acts not only by exporting the antimicrobial into the extracellular environment, but also by protecting the target lipid II, and cell wall modification (Reiners *et al.*, 2017; Gottstein *et al.*, 2022).

In this study we analysed three parameters relating to nisin immunity and resistance proteins; (i) the effect of each protein-based nisin immunity and resistance mechanisms on nisin sensitivity as deduced from MIC assays, (ii) the impact that each nisin defense system has on the growth of nisin sensitive strains (MG1614), strains which already confer protection to nisin via immunity proteins (NZ9800/NZ9700) or strains containing resistance proteins (MG1614 pNP40), and (iii) the effect of increased nisin immunity on nisin production in the NZ9700 strain. Previous research has examined the roles of NisI and NisFEG when expressed in separate vectors and their effect on immunity to nisin A (Siegers and Entian, 1995; Takala and

Saris, 2006; Lagedroste *et al.*, 2019b), but to the best of our knowledge, this is the first study in which both genes are co-expressed within the same high-copy vector. It is worth noting that all strains in this study are derivatives of MG1363: MG1614, NZ9700 (a nisin-producing transconjugant) and NZ9800 (a derivative of NZ9700 in which the *nisA* gene has been inactivated (Δ*nisA*) (Kuipers *et al.*, 1993).

Our results demonstrate that NisI and NisFEG both provide essentially the same level of resistance to nisin A, in agreement with that reported by Reiners and co-workers for NisI and NisFEG expressed in the NZ9000 strain (Reiners *et al.*, 2020). No strain harbouring immunity or resistance determinants on pNZ44, including NZ9800, was capable of imparting immunity from nisin A to resemble that of the native nisin producer, NZ9700 (15 µM) (Table 3). Indeed, it has been demonstrated previously that the NZ9800 strain exhibits a 10-fold decrease in protection to exogenously applied nisin (Oscar P. Kuipers *et al.*, 1993), in agreement with the findings of this study where we observed an 8-fold reduction compared to the NZ9700 wild-type strain (Table 3). The Kuipers study also found that the immunity of NZ9800 was fully restored following complementation with plasmids harbouring *nisA* and *nisZ* genes under control of the efficient *lac* promoter, enabling production of nisin A or nisin Z, respectively. This finding, in addition to the results of this study, confirms that the NZ9800 strain is incapable of exhibiting full protection in comparison to the native nisin-producing strain, probably due to a lack of nisin production and subsequent induction since transcription of *nisA* is dependent on the integrity of *nisA* itself (Kuipers *et al.*, 1993). Remarkably, a genetically-modified NZ9800 strain with a deletion in the *nisP* gene to render a Δ*nisA* and Δ*nisP* variant, designated NZ9803 exhibited a >1000-fold increase in immunity to nisin A (when pre-induced with nisin) in comparison to the nisin sensitive strain NZ9000 (Deng, 2020). In this study, induction of the NZ9800 strain was carried out at 50 ng mL^{-1} with nisin M, a bioengineered nisin A variant which has been shown to exhibit little antimicrobial activity whilst retaining the same level of

induction capacity as the wildtype peptide (O'Connor *et al.*, 2020). Surprisingly, NZ9800 immunity was not increased under these conditions (Table 5). In contrast, NZ9700 can protect itself from approximately 10-fold the concentration of nisin it is capable of producing (5.3 µg mL $^{-1}$) and to the equivalent of >960 fold that of MG1614, as shown in the AUC assays discussed later. However, the ability to increase nisin immunity by increased copy numbers of immunity genes in NZ9800 was observed with a two-fold increase in immunity provided by extra copies of NisI and NisFEG, and a four-fold increase observed when both proteins are overexpressed (Table 5). This immunity, however, still did not match that of the nisin-producing strain NZ9700 (Table 1 and Table 6).

Transformants of NZ9700 harbouring the nisin immunity genes alone or in combination offered a mixed set of results. A two-fold increase in immunity was observed for NZ9700 pNZ44*nisI*, while NZ9700pNZ44*nisFEG* brought about a two-fold reduction in immunity compared to the control (Table 6). We speculate that this is due to the action of NisFEG in exporting nisin from the cell membrane and therefore severely diminishing the induction activity of nisin. Therefore, based on these results it can be assumed the majority of the immunity observed in the NZ9700 pNZ44*nisFEG* strain is most likely due to the action of NisFEG which subsequently reduces the induction of the entire nisin operon, including chromosomal immunity genes.

Notably, growth of the MG1614 strains carrying pNZ44*nsr* and pNZ44*nsrFP* was negatively impacted in terms of maximum OD reached ($p < 0.05$), as well as increasing the lag phase of growth compared to the control, MG1614 ($p < 0.0001$) (Figures 2 and 3) and suggests a significant cost to the cell to achieve the 30-fold and 120-fold higher resistance compared to the wild type strain, respectively (Table 3). This observation is replicated for MG1614 harbouring pNZ44*nsr* which provides two-fold increased resistance when compared to MG1614 with pNP40 (Table 4) and where a significant difference in growth rate ($p < 0.05$),

maximum OD ($p < 0.005$) and lag phase ($p < 0.0001$) was observed (Figure 3). These negative impacts on growth could be a result of the expression of *nsr* controlled by the strong, constitutive P44 promoter in the pNZ44 plasmid, however, the promoter associated with *nsr* on the plasmid pNP40 has not yet been characterised.

Notably, a difference was apparent across all three aspects analysed in growth curves between the MG1614 strain harbouring pNP40 and MG1614 harbouring pNZ44*nsr,* (Figure 3), suggesting that the overexpression of Nsr on pNZ44 has an overall negative effect on growth, in contrast to the pNP40 strain. In contrast, the growth of MG1614 harbouring both pNP40 and pNZ44*nsr* remained comparable in both growth rate and lag phase to the control (Figure 3). This was unexpected given the four-fold increased resistance to the strain harbouring pNP40 alone (Table 4). Accordingly, it was expected that MG1614 harbouring both pNP40 and pNZ44*nsrFP* would also exhibit enhanced resistance given that both these resistance determinants often co-occur within operons in pathogenic strains (Khosa *et al.*, 2013). However, the MIC remained comparable to MG1614 pNP40 (Hayes *et al.*, 2019). This finding suggests that the coordinated expression of NsrFP with Nsr does not work cooperatively (Table 4) and warrants further investigation.

Maximum cell density in growth curves of nisin-producing strains revealed significant differences between control and NZ9700 harbouring the pNZ44*nisIFEG* plasmid (Figure 5). While this result suggests a negative effect due to the presence of increased copies of both immunity proteins it was determined there was no negative effect on the immunity exhibited by the strain in terms of MICs (Table 6). Interestingly, NZ9700 harbouring pNZ44*nisI* displayed a significant difference to the control NZ9700 strain whereby a greater than two-hour delay in attaining exponential growth was observed ($p < 0.05$) (Figure 5) and is likely linked to the burden associated with the enhanced immunity phenotype observed for this strain (Table 6).

Nisin production

Several factors surrounding nisin production have been optimised to generate very pure nisin, for example, the >95% pure NisinA®P and NisinZ®P products from Handary, though some limitations including stability, yield and expense still persist (Khelissa, Chihib and Gharsallaoui, 2021). Indeed, studies have been conducted relating to nisin immunity and its role in improving nisin production and yield. One such study reported a 44% increase in nisin production by an *L. lactis* strain (FL-75) (Dzhavakhiya *et al.*, 2018) following exposure to increasing concentrations of bacitracin rendering it resistant to both nisin and bacitracin. However, the authors provided no details regarding the genetic changes within the strain and also reported the increased production based on activity exhibited (AU/ml and U/10^9) and not specific nisin concentrations *via* standardised AUC tests.

Numerous studies have been published highlighting increased nisin production in strains harbouring extra copies of the structural gene as well as the two-component sensory/regulatory system and immunity genes (Cheigh *et al.*, 2005; Hu *et al.*, 2010; Ni *et al.*, 2017; Portieles *et al.*, 2023). For example, cloning of the *nisA*, *nisRK* and *nisFEG* genes together under control of the constitutive P32 promoter (Ni *et al.*, 2017) is reported to have brought about an increase in nisin production by 66.3% and 52.6% in shake flasks and 1L fermenters, respectively. However, it was also reported that the level of transcription of *nisE* decreased in this strain suggesting that the increased nisin production observed was due to the extra copies of NisRK and NisA (Ni *et al.*, 2017). Moreover, the *L. lactis* strain utilised in this study, LS01, had already been genetically modified via chemical and physical mutagenesis to produce higher levels of nisin than the parental strain (Ni *et al.*, 2017). Similarly, Cheigh and coworkers cloned the *nisRK* and *nisFEG* genes for nisin Z separately under control of a strong lactococcal promoter (pOri23) and following introduction into an *L. lactis* nisin Z producer, observed an equivalent increase in production for both *L. lactis* pOri23*nisRK* and *L. lactis* pOri23*nisFEG*

when analysed *via* northern blot analysis and a critical microdilution method reported as AU mL^{-1} (Cheigh *et al.*, 2005). Significantly, the authors noted that the strain harbouring pOri23*nisFEG* produced nisin more slowly than the pOri23*nisRK* derivative, despite no observed difference in growth rate for the control or transformants and postulated that the increased immunity raised the host-specific ceiling concentration by efficiently reducing the intracellular nisin concentration, thereby contributing to raising the final level of exported nisin (Cheigh *et al.*, 2005).

NisI has also been the target in improved nisin production-related studies. For instance, a construct was generated whereby the nisin Z structural gene was placed under the control of the strong, constitutive promoter (P32), followed by subsequent cloning of the *nisI* gene under the P59 strong constitutive promoter resulting in the pHMI plasmid which was transformed into NZ9800 (Hu *et al.*, 2010). The strain harbouring pHMI was found to exhibit greater inhibition against a *Micrococcus* indicator in comparison to the control (harbouring *nisZ* alone) with a 32% and 25% increase in antimicrobial activity observed after 8 and 6 hours of fermentation, respectively. Moreover, no impact on growth rates was observed in agreement with our analysis of NZ9700 harbouring pNZ44*nisI* and the control NZ9700 (Figure 5). Notably, the authors reported a 25% increase in immunity in NZ9800 pHMI while we observed a two-fold (100%) increase in immunity via MIC assays (Table 6). These contrasting results between this study and the work carried out by Hu and co-workers (2010) could be due to the difference in nisin peptide (nisin A and nisin Z) or the methods used to quantify nisin production or choice of indicator strains.

Here, we monitored nisin production with three different assays; DAAs of the NZ9700 wild type and its transformants against a standard indicator (*L. lactis* HP), well diffusion assays (WDA) of cell-free-supernatant (CFS) from these same NZ9700 strains against the same indicator, and area under the curve (AUC) analysis to quantify nisin production based on a

standard curve created with pure nisin A peptide. Our results demonstrate across all three assays that increasing the number of nisin immunity proteins in the cell had no positive effect on peptide production in the NZ9700 strain. No statistically significant difference in nisin production was observed in strains harbouring empty pNZ44 vector and pNZ44*nisI* when compared to the control. An overall negative effect on nisin production was observed for NZ9700 with pNZ44*nisFEG* for all assays performed with significant differences in DAA ($p < 0.0001$), CFS ($p < 0.005$) (Figure 6A & B) and AUC ($p < 0.005$) or pNZ44*nisIFEG* in AUC analysis ($p < 0.005$) (Figure 7A-C). MIC results confirmed the increased immunity of the nisin-producing strain harbouring pNZ44*nisI* in comparison to the control (Table 6), suggesting increased immunity does not equate to increased production. However, the immunity of NZ9700 pNZ44*nisFEG* was reduced to half that of the control (Table 6). Furthermore, the strain could produce nisin as observed in overlay assays (Figure 6A), most likely due to the induction of nisin production between closely situated cells on solid media. Given that the NZ9700 pNZ44*nisFEG* strain was capable of producing nisin on solid media but not in the CFS, this decline in peptide production could relate to the decreased immunity observed by the strain (Table 6). Indeed, it is likely that the concentration of nisin that is critical for induction of the operon via NisRK is unable to reach the concentration required for full autoinduction of the operon including chromosomal *nisFEG* since membrane-bound NisFEG derived from pNZ44*nisFEG* is already functioning to export nisin from the membrane. We supported this hypothesis by induction of the NZ9700 pNZ44*nisFEG* strain with nisin M (O'Connor *et al.*, 2020) at 50 ng mL^{-1}. Following induction and overnight growth, the CFS from induced NZ9700 pNZ44*nisFEG* PT was bioactive and uninduced strains were devoid of bioactivity (Figure 6B). This hypothesis would need to be tested further with induction of the strain as described above, followed by AUC analysis.

Based on these results it can be assumed immunity and production do not share any linear

correlation, where increased immunity does not automatically result in increased production (Table 6 & Figure 7A-C). Additionally, while there were significant differences in the carrying capacity of the NZ9700 strains harbouring pNZ44, pNZ44*nisI* ($p < .0001$) and pNZ44*nisFEG* ($p < .005$), and a difference in the lag phase between NZ9700 pNZ44*nisI* and control ($p < .05$) (Figure 4), there was no significant difference in growth rate between the control and all NZ9700 test strains in this assay, including the pNZ44*nisFEG* clone with decreased antimicrobial production (Figure 7A-C).

To fully understand the relationship between immunity and production a set of gene knockouts could be created within the *L. lactis* NZ9700 strain, where proteins NisI, NisFEG and both NisI and NisFEG would be incapable of being translated, and one could observe the effect of each knockout on immunity and production in a natural background.

While the enhancement of nisin production and yield is still very desirable, it has been suggested there is too broad a range of methodologies in the quantification of nisin across studies attempting to do so, making it more difficult for comparisons to be made (Khelissa, Chihib and Gharsallaoui, 2021), and implying that methods to specifically analyse nisin production should be standardised and employed going forward.

The results from this study also have particular significance in relation to antimicrobial resistance especially given recent reports highlighting the prevalence and abundance of lantibiotic resistance system genes amongst a collection of over 700 unique human gut organisms. Indeed, it was reported that almost 35% of these bacteria harboured *lanFEG* and/or *lanI* resistance determinants, especially in the Bacillota (previously Firmicutes) while both *lanA* and *lanBC* were detected in <1% (Zhang, Cole, *et al.*, 2023). Moreover, recent findings suggest that BceAB-type transporters such as NsrFP are widely distributed amongst human pathogenic bacteria (Gottstein, 2023). Consequently, the occurrence and dissemination of

resistance genes such as those described in this study above across multiple species including important human pathogens poses a significant challenge for the potential use of nisin and other bacteriocins in therapeutic applications.

Conclusion

The aim of this study was to add to the knowledge of nisin immunity and resistance proteins, NisI, NisFEG, Nsr and NsrFP using sensitivity assays (MIC), growth curve analysis and their impact on nisin production using overlays, WDAs and AUC assays. The results reveal that NisI and NisFEG work co-operatively when expressed together as previously reported, but unlike previous studies, have not been expressed in combination in a plasmid system until now. Moreover, the ability to increase nisin immunity by provision of extra copies of NisI in the nisin-producing strain NZ9700, or resistance with extra copies of Nsr in MG1614 with pNP40 was confirmed. Although the aim to increase nisin production via implementation of extra copies of immunity genes and therefore raising the protection of NZ9700 from the action of nisin was not achieved in this study, the results obtained do provide additional information regarding the relationship between immunity and its impact on production. It can be concluded that there is no direct correlation between increasing nisin immunity and increasing production in the NZ9700 strain. It can also be concluded that while significant and excellent, research on immunity and resistance mechanisms in lantibiotics is ongoing, and in particular for nisin; there is still a lack of knowledge on exactly how these proteins work co-operatively in providing immunity to producing strains.

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Tables and Figures

Table 1. Strains and plasmids used in this study.

Strain	Characteristic	Reference
E. coli Top10	Intermediate cloning host	Invitrogen
L. lactis MG1614	RifR StrpR *L. lactis* derivative of MG1363. Nisin sensitive strain (Imm^-). Non-nisin producing strain (Nis^-).	(Gasson, 1983)
L. lactis NZ9700	Progeny of the conjugation between nisin producer strain NIZO B8 with MG1614. Nisin immune strain (Imm^+). Nisin producing strain (Nis^+).	(Oscar P. Kuipers *et al.*, 1993)
L. lactis NZ9800	Derivative of NZ9700 (NIZO B8 & MG1614) with 4bp deletion rendering an inactive nisin operon, except *nisRK* (Δ*nis*A). Nisin immune (Imm^+). Non-nisin producing strain (Nis^-).	(Oscar P. Kuipers *et al.*, 1993)
L. lactis subsp. cremoris HP	Nisin sensitive indicator	UCC Culture Collection
Plasmids	Characteristic	Reference
pNZ44	High copy plasmid pNZ8048 with deletion of P*nisA* promoter replaced with constitutive P44 promoter from *L. lactis* chromosome.	(McGrath, Fitzgerald and Van Sinderen, 2001)

Table 1. continued

pNP40	Naturally occurring conjugative lactococcal plasmid carrying nisin resistance gene, *nsr*	(McKay and Baldwin, 1984; Field *et al.*, 2019)
pNZ44*nisI*	pNZ44 plasmid harbouring nisin immunity gene, *nisI*.	This study
pNZ44*nisFEG*	pNZ44 plasmid harbouring nisin immunity gene, *nisFEG*.	This study
pNZ44*nisIFEG*	pNZ44 plasmid harbouring nisin immunity genes, *nisIFEG*.	This study
pNZ44*nsr*	pNZ44 plasmid harbouring nisin resistance gene, *nsr*.	This study
pNZ44*nsrFP*	pNZ44 plasmid harbouring nisin resistance gene, *nsrFP*.	This study

Table 2. Primers used for cloning in this study.

Primer name & sequence (5' - 3')		Clone associated with
Forward	**Reverse**	
NcoI-RBS-nisFEG-For CGGCCATGGAAGGAGGGTGAGTGTA TGCAGGTAAAAATTCAAAATCTTTC	**HindIII-nisFEG-Rev** GCCAAGCTTTTATCTAATCTT TTTTTTAGATAATGCTACAAG	pNZ44 *nisFEG*
NcoI-RBS-nisI-For CGGCCATGGTAAGGAGGCACTCACA ATGAGAAGATATTTAATACTTATT	**NcoI-nisI-Rev** GCCCCATGGCTAGTTTCCTAC CTTCGTTGC	pNZ44 *nisIFEG*
NisI-RBS For TAAGGAGGCACTCACAATGAGAAG ATATTTAATACTTATT	**NisI-Rev** CTAGTTTCCTACCTTCGTTGC	pNZ44 *nisI*
pNZ44NisIFor(NcoI) **TATGGCCAAACCATG**TAAGGAGGC ACTCACAATGAGAAG	**NisIpNZ44Rev(HindIII)** **GGTTCAAAGAAAGCT**CTAG TTTCCTACCTTCGTTGCAAGC	

Table 2. continued

NSRDRC3RBSNcoIFor	**NSRDRC3RevXbaI**	pNZ44
AATTCCATGGTAAGGAGGCACTCACAAT GAAAATAGGTAAGCGCATT	GGTCTAGATTACTTTATT TGAGATTTTATC C	*nsr*
NsrFP-RBSNcoIFor	**NsrFP-RevPstI**	pNZ44
AATTCCATGGTAAGGAGGCACTCACAAT GTTATTAGAAATCAATCACTTAG	AATTCTGCAGTTAGCGT TCAATAATATGATA	*nsrFP*

Underlined bases indicate restriction site in primers. Bases marked in **bold** in pNZ44NisI For & Rev primers indicate bases complementary to pNZ44 plasmid for infusion cloning tool (see https://www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools)

Table 3. Specific activity of nisin A vs *L. lactis* MG1614 harbouring nisin immunity/resistance genes on the plasmid pNZ44 to determine which immunity mechanism offers more protection to cells.

Strain	**Nisin A μg mL^{-1} (μM)**	**Protection conferred compared to MG1614**
L. lactis MG1614	0.052 μg ml^{-1} (0.0156)	N/A
L. lactis MG1614 pNZ44	0.052 μg ml^{-1} (0.0156)	N/A
L. lactis MG1614 pNZ44*nisI*	3.125 μg ml^{-1} (0.938)	>60X
L. lactis MG1614 pNZ44*nisFEG*	3.125 μg ml^{-1} (0.938)	>60X
L. lactis MG1614 pNZ44*nisIFEG*	6.25 μg ml^{-1} (1.875)	>120X
L. lactis MG1614 pNZ44*nsr*	1.563 μg ml^{-1} (0.468)	30X
L. lactis MG1614 pNZ44*nsrFP*	6.25 μg ml^{-1} (1.875)	>120X
L. lactis NZ9800	6.25 μg ml^{-1} (1.875)	>120X
L. lactis NZ9700	50 μg ml^{-1} (15)	>960X

Table 4. Specific activity of nisin A vs *L. lactis* MG1614 harbouring nisin resistance genes on the plasmid pNZ44 to determine which resistance mechanism offers more protection to cells.

Strain	Nisin A μg mL^{-1} (μM)	Times increase in resistance compared to MG1614 pNP40
L. lactis MG1614 +/- pNZ44	0.052 μg ml^{-1} (0.0156)	N/A
L. lactis MG1614 pNP40	0.78 μg ml^{-1} (0.234)	N/A
L. lactis MG1614 pNP40 pNZ44	0.78 μg ml^{-1} (0.234)	0X
L. lactis MG1614 pNZ44*nsr*	1.563 μg ml^{-1} (0.468)	2X
L. lactis MG1614 pNZ44*nsrFP*	6.25 μg ml^{-1} (1.875)	>8X
L. lactis MG1614 pNP40 + pNZ44*nsr*	3.125 μg ml^{-1} (0.938)	>4X
L. lactis MG1614 pNP40 + pNZ44*nsrFP*	0.78 μg ml^{-1} (0.234)	0X

Table 5. Specific activity of nisin A vs *L. lactis* NZ9800 harbouring nisin immunity genes on the plasmid pNZ44 to determine if immunity can be increased in a strain already harbouring nisin protection proteins on the genome. PT denotes pre-treatment with nisin M.

Strain	**Nisin A µg mL^{-1} (µM)**	**Increased immunity compared to NZ9800**
NZ9800	6.25 µg ml^{-1} (1.875)	N/A
NZ9800 PT 50 ng mL^{-1}	6.25 µg ml^{-1} (1.875)	0X
NZ9800 pNZ44	1.563 µg ml^{-1} (0.468)	-4X
NZ9800 pNZ44*nisI*	12.5 µg ml^{-1} (3.75)	2X
NZ9800 pNZ44*nisFEG*	12.5 µg ml^{-1} (3.75)	4X
NZ9800 pNZ44*nisIFEG*	25 µg ml^{-1} (7.5)	4X

Table 6. Specific activity of nisin A vs *L. lactis* NZ9700 harbouring nisin immunity genes on the plasmid pNZ44 to determine if immunity can be increased in a nisin-producing strain already harbouring nisin protection proteins on the genome.

Strain	**Nisin A µg mL^{-1} (µM)**	**Increased immunity compared to NZ9700**
NZ9700	50 ug.ml^{-1} (15)	N/A
NZ9700 pNZ44	50 ug.ml^{-1} (15)	0X
NZ9700 pNZ44*nisI*	100 ug.ml^{-1} (30)	2X
NZ9700 pNZ44*nisFEG*	25 ug.ml^{-1} (7.5)	-0.5X
NZ9700 pNZ44*nisIFEG*	50 ug.ml^{-1} (15)	0X

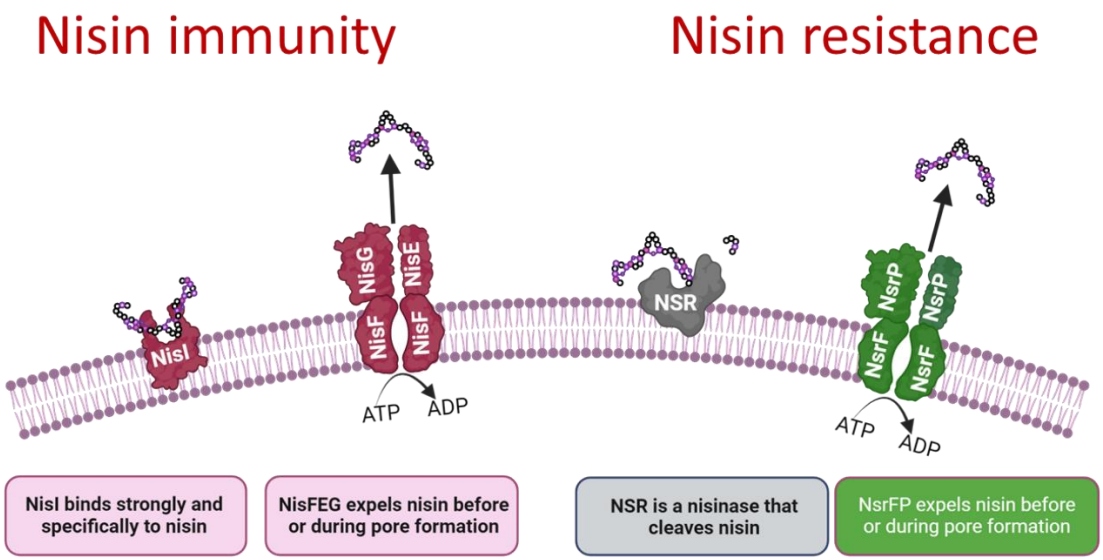


Figure 1. Schematic representation of nisin immunity (NisI and NisFEG) and nisin resistance (Nsr and NsrFP) mechanisms. Image originally published by Field *et al.*, 2023

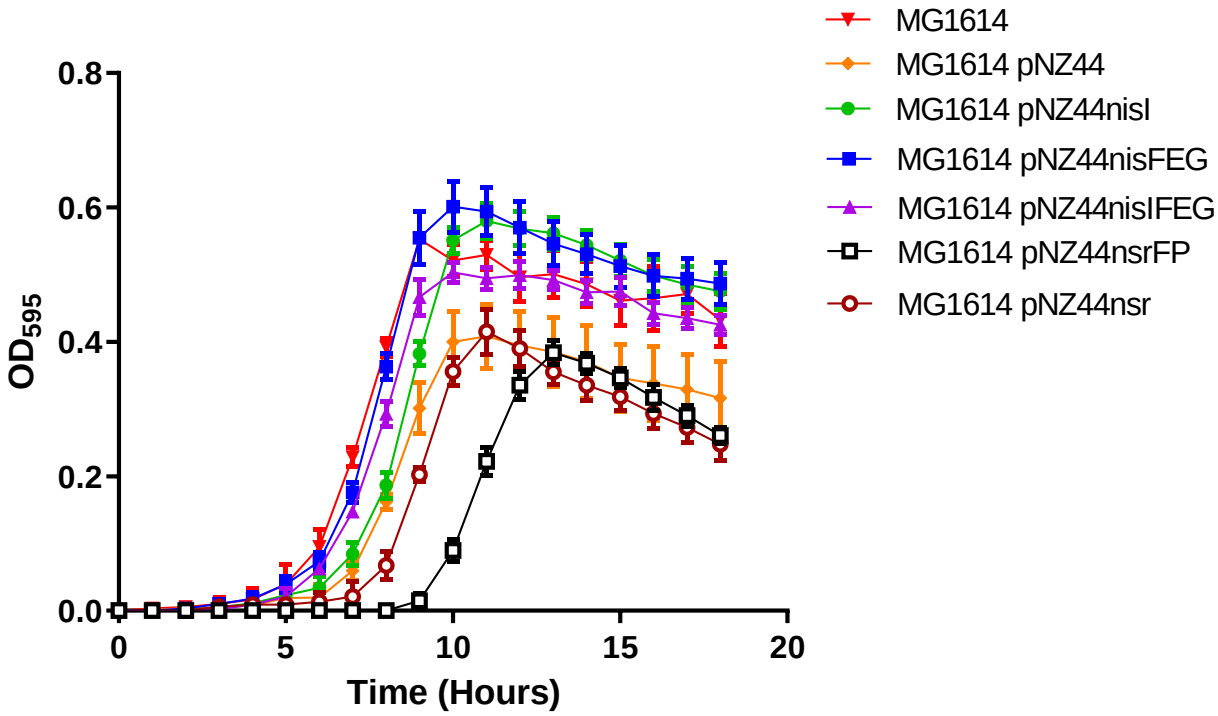


Figure 2. Growth curve of *L. lactis* MG1614 strains harbouring the nisin immunity and resistance protein genes, *nisI*, *nisFEG*, *nisIFEG*, *nsr* and *nsrFP* on the high copy plasmid, pNZ44. Control strain is MG1614 with no plasmid (red upside-down triangles).

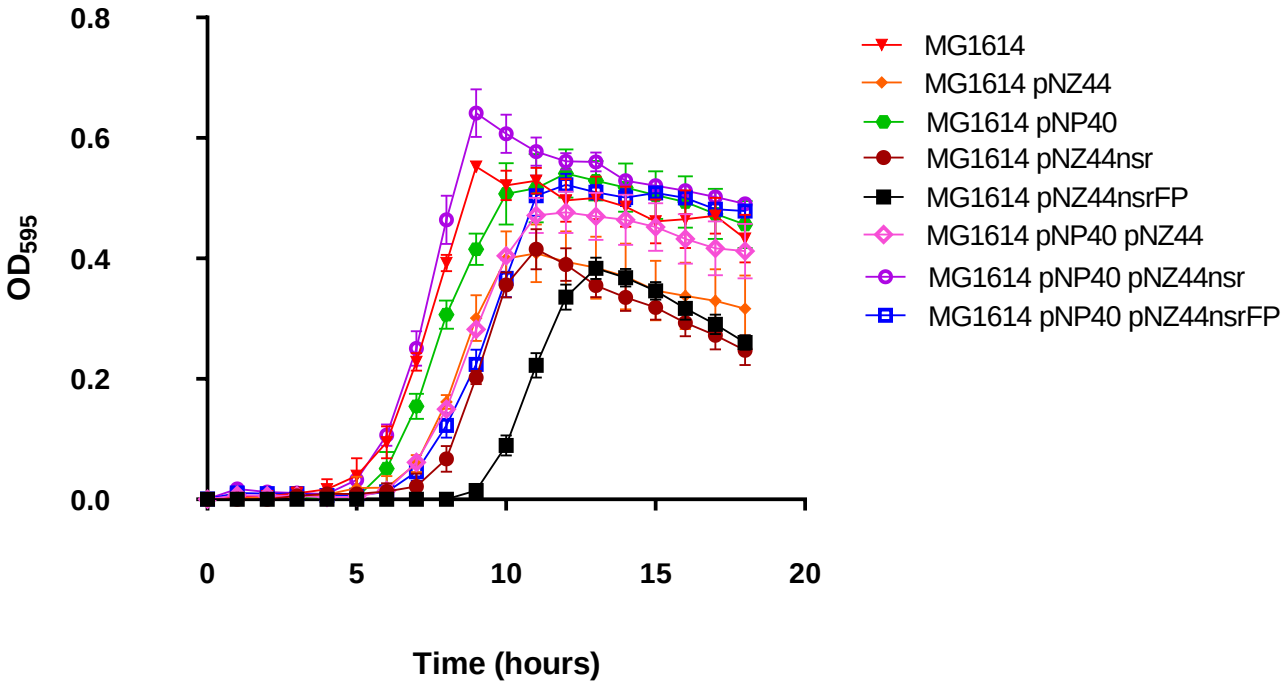


Figure 3. Growth curves of *L. lactis* MG1614 strain harbouring the natural nisin resistance plasmid, pNP40 (*nsr*+) with and without increased resistance mechanisms via the nisin resistance proteins, Nsr and NsrFP harboured on the pNZ44 plasmid. Controls include the MG1614 strain with no vector (red upside-down triangles).

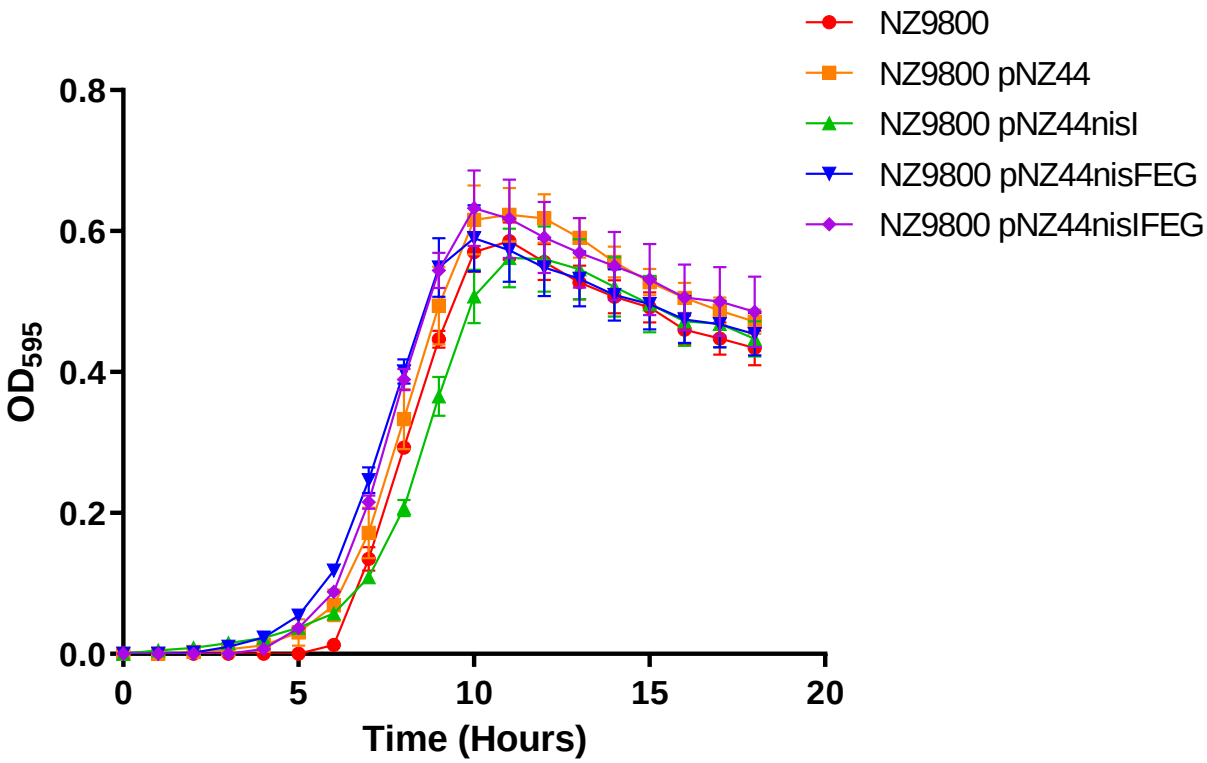


Figure 4. Growth curve of nisin immune *L. lactis* NZ9800 both with and without increased copies of immunity genes on the pNZ44 vector. Controls for this growth curve are the NZ9800 strain without plasmid (red circles)

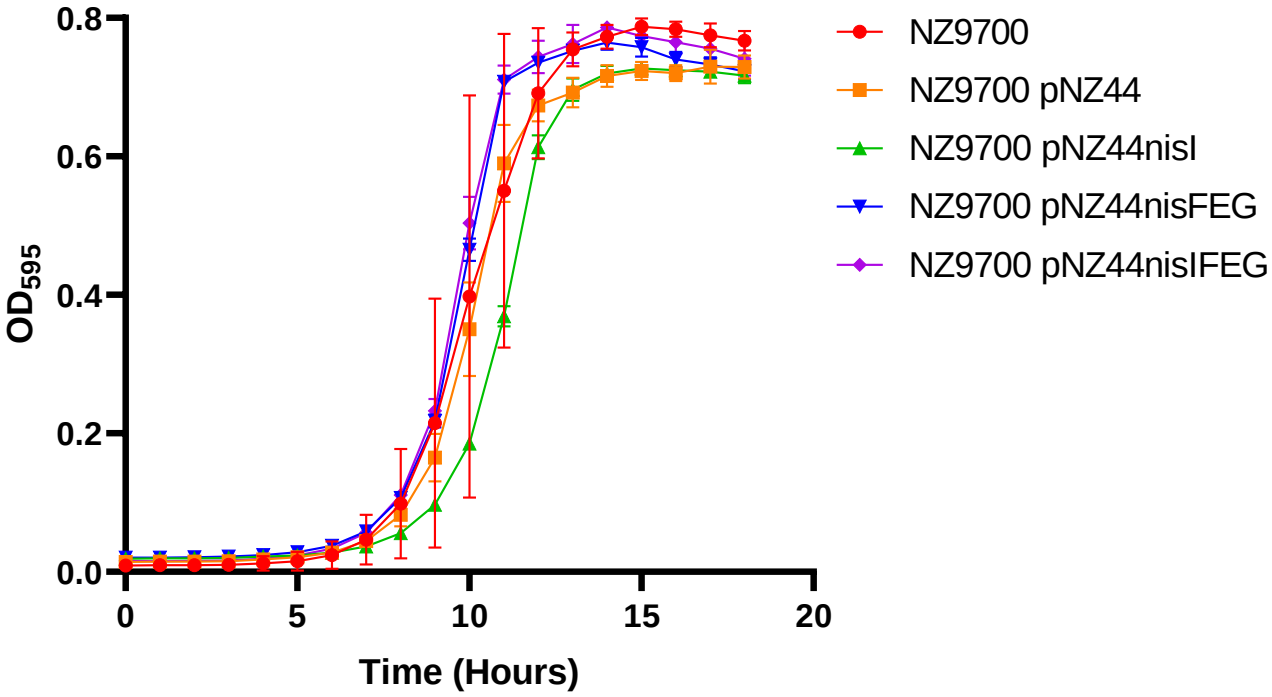


Figure 5. Growth curve of nisin producing and nisin immune *L. lactis* NZ9700 both with and without increased copies of immunity genes on the pNZ44 vector. Controls for this growth curve are the NZ9700 strain without plasmid (red circles)

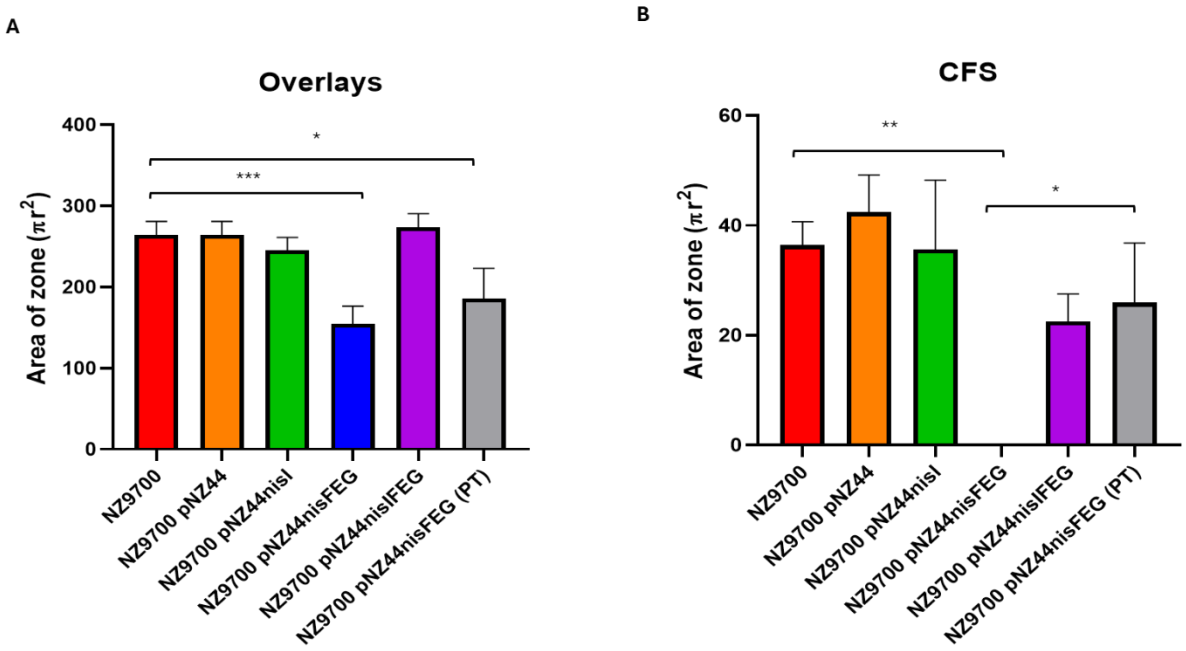
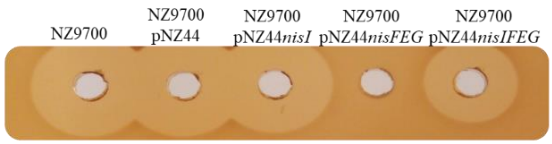


Figure 6. Column graphs of nisin quantifications via measurements of zones exhibited by *L. lactis* NZ9700 with and without pNZ44 harbouring nisin immunity genes against *L. lactis* HP indicators in (A) overlay assays and (B) cell-free supernatant (CFS). Asterisk (*) denotes where statistical differences were observed between the test strains and control (overlays and CFS), and test strains harbouring pNZ44*nisFEG* induced and uninduced with nisin M (CFS only) (*** = p <0.0001; ** = p <0.005; * = p <0.05). PT denotes pre-treatment with nisin M.

A



B

Strain	Nisin concentration (µM)	Concentration difference to NZ9700	% nisin concentration
NZ9700	5.3 µg ml^{-1} (1.59)	N/A	100%
NZ9700 pNZ44	7.2 µg ml^{-1} (2.17)	+1.9 µg ml^{-1}	137%
NZ9700 pNZ44*nisI*	5.5 µg ml^{-1} (1.66)	+0.07µg ml^{-1}	104%
NZ9700 pNZ44*nisFEG*	0**	-5.3 µg ml^{-1}	0%
NZ9700 pNZ44*nisIFEG*	1.53 µg ml^{-1} (0.49)**	-3.77 µg ml^{-1}	31%

C

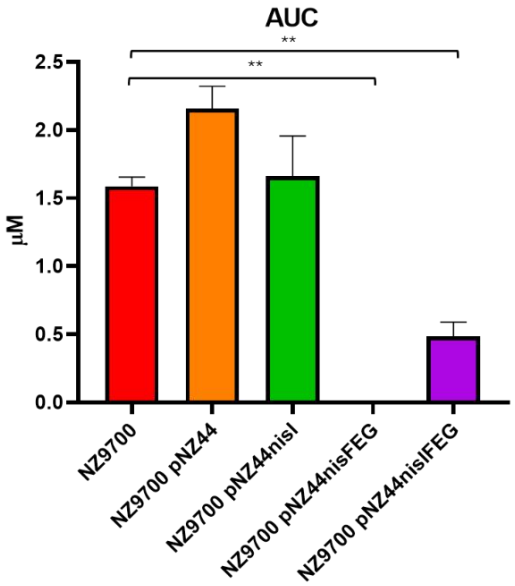
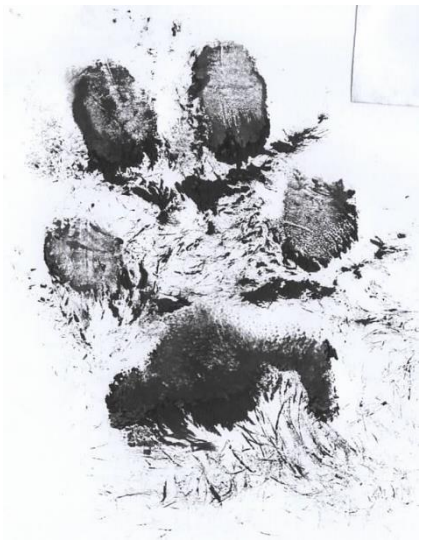


Figure 7. Area under the curve analysis of *L. lactis* NZ9700 harbouring increased copies of nisin immunity genes on the pNZ44 vector. (**A**) WDA of CFS analysed for nisin quantification against *L. lactis* HP. (**B**) Specific nisin concentrations for each strain measured via mV response on a standard curve of nisin A. (**C**) Visualisation of specific nisin concentrations measured in table B in column graph format. Asterisks (*) in (**A**) denotes statistical differences between nisin concentration of these strains in comparison to the control, NZ9700. Asterisks in both (**B**) and (**C**) denote where statistical differences were observed between test strains and the control, NZ9700 harbouring no vector. (** = p <0.005).

Chapter 3. Nisin M: a bioengineered Nisin A variant that retains full induction capacity but has significantly reduced antimicrobial activity.



Bailey

Abstract

Nisin A is a potent antimicrobial with potential as an alternative to traditional antibiotics, and a number of genetically modified variants have been created that target clinically relevant pathogens. In addition to antimicrobial activity, nisin auto-regulates its own production via a signal transduction pathway, a property that has been exploited in a protein expression system termed the Nisin Controlled Gene Expression (NICE) system. Although NICE has become one of the most popular protein expression systems, one drawback is that the inducer peptide, nisin A, also has inhibitory activity. It has already been demonstrated that the N-terminal region of nisin A contributes to antimicrobial activity and signal transduction properties, therefore, we conducted bioengineering of nisin at positions Pro9 and Gly10 within ring B to produce a bank of variants that could potentially be used as alternative induction peptides. One variant, designated nisin M, has threonines at positions 9 and 10 and retains induction capacity comparable to the wild type nisin A, while most of the antimicrobial activity is abolished. Further analysis confirmed that nisin M produces a mix of peptides as a result of different degrees of dehydration of the two threonines. We show that nisin M exhibits potential as a more suitable alternative to nisin A for the expression of proteins that may be difficult to express, or to produce proteins in strains that are sensitive to wild type nisin. Moreover, it may address the increasing demand by industry for optimization of peptide fermentations to increase yields or their production rate.

Importance

This study describes the generation of a nisin variant with superior characteristics for use in the NICE protein expression system. The variant, termed nisin M, retains an induction capacity comparable to the wild type nisin A but exhibits significantly reduced antimicrobial activity and can therefore be used at concentrations that are normally toxic to the expression host.

Introduction

Producing high quantities of proteins of biotechnological and pharmaceutical value from their natural sources can have economic challenges. Although *Escherichia coli* has been the dominant player in the production of recombinant proteins for decades, several issues including the presence of endotoxin or lipopolysaccharide requires expensive and often problematic downstream purification processes (Cano-Garrido *et al.*, 2014). The lactic acid bacteria (LAB) *Lactococcus lactis* has gained importance as a host for heterologous protein expression due to its well understood genetics and metabolism, generally regarded as safe (GRAS) status, as well as the availability of a wide range of genetic tools. Indeed, a major advance with regards to protein expression in *L. lactis* was the discovery and use of gene expression systems based on a number of inducible promoters. These include promoters that respond to the environment such as P170, which is upregulated at low pH (Madsen *et al.*, 1999) and zinc-based systems that respond to zinc availability (Llull and Poquet, 2004). One of the best known and most widely employed expression systems is the nisin-inducible controlled gene expression (NICE) system (Mierau and Kleerebezem, 2005; Mierau *et al.*, 2005) which stems from the nisin biosynthetic operon (*nisABTCIPRKFEG)* found in some *L. lactis* strains (Kuipers *et al.*, 1995). Nisin is a 34 amino acid peptide and is the most extensively studied bacteriocin (ribosomally synthesized, antimicrobial peptides produced by bacteria) (Wirawan *et al.*, 2006; Field *et al.*, 2008). It targets a wide range of Gram-positive bacteria, including food pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and clostridia (Naghmouchi *et al.*, 2010; D Field *et al.*, 2015). Nisin induces its own biosynthesis via a two-component signal transduction pathway NisRK (Kuipers *et al.*, 1995) and has led to the development and application of a food grade expression system using *L. lactis* as the host (De Ruyter, Kuipers and De Vos, 1996). The NICE system encompasses both regulatory elements of the nisin operon, P*nisA*, the nisin-inducible

promoter (cloned into several expression vectors) and *nisRK*, the two-component histidine kinase response regulator system (harboured by compatible plasmids or inserted on the chromosome of a suitable host strain). The system is 'switched on' by the addition of nisin in the nanomolar range which activates the receptor NisK. NisK activates NisR by phosphorylation and the activated NisR induces expression at the nisin A promoter (Mierau and Kleerebezem, 2005). The NICE system has been extensively used to produce proteins in *L. lactis*, such as bacteriophage lysins and metalloendopeptidases to demonstrate their potential in dairy fermentations (de Ruyter *et al.*, 1997; Hickey, Ross and Hill, 2004). Moreover, NICE can, under certain conditions and with some modifications to the system components, also be used in other species of LAB and in other Gram-positive bacteria (De Ruyter, Kuipers and De Vos, 1996; Kleerebezem *et al.*, 1997). Its numerous advantages include ease of use, exquisitely controlled and efficiently induced expression and amenability to large-scale production processes. As an example, nisin induced fermentations of the antimicrobial lysostaphin have been carried out and even identified areas of the NICE system that need improvement (Mierau *et al.*, 2005). However, for industrial applications, nisin addition remains costly(Özel *et al.*, 2018). Another drawback of the system is that the inducing peptide is also toxic due to its potent antimicrobial activity. Therefore, a nisin peptide that retains its induction capacity whilst having little to no antimicrobial activity would be highly desirable. The gene-encoded nature of the nisin peptide makes genetic engineering to develop certain characteristics of the molecule an attractive and feasible option. Although the bioengineering of nisin commenced over three decades ago, the majority of studies have largely focused on identifying nisin variants with enhanced antimicrobial activity or an extended-antimicrobial spectrum (D Field *et al.*, 2015; Ge *et al.*, 2016; Zhou *et al.*, 2016). The importance of the N-terminus rings A and B with respect to induction has been highlighted on a number of occasions (Kuipers *et al.*, 1995; Rink *et al.*,

2007; Ge *et al.*, 2016). These studies involved either combinatorial saturation mutagenesis of rings A and B (Rink *et al.*, 2007) or the application of alanine scanning approaches to assess the antimicrobial activity and induction properties of various nisin derivatives (Ge *et al.*, 2016).

In this study, we carried out a more comprehensive bioengineering approach and created banks of nisin derivatives that have been randomized at positions 9 (P9X) and 10 (G10X) individually and in combination (P9XG10X) and assessed them for antimicrobial activity in conjunction with their ability to induce the nisin promoter using GFP and β-galactosidase reporter systems.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 3.

Creation and analysis of a bank of nisin A ring-B derivatives

Mutagenesis of the *nis*A gene was carried out as described previously (Field *et al.*, 2008). Briefly, saturation mutagenesis was carried out using pDF05 (pCI372-*nisA*) as template and using oligonucleotides as listed in (Table 4) containing an NNK codon in place of each native codon. PCR amplification was performed in a total volume of 50 μL with 0.5 ng of target DNA (pCI372-*nisA*), 1 unit Phusion High-Fidelity DNA polymerase (Finnzymes, Finland), 1 mM dNTPs and 500 ng each of the appropriate forward and reverse oligonucleotides. The reaction was pre-heated at 98°C for 2 min, and then incubated for 29 cycles at 98°C for 30 s, 55°C for 15 s and 72°C for 3 min 30 s, and then finished by incubating at 72°C for 3 min 30 s. Amplified products were treated with Dpn1 (Stratagene) for 60 min at 37°C to digest template DNA and purified using the QIAquick PCR purification kit. Following transformation of *E. coli* Top 10 cells plasmid DNA was isolated and sequenced using primers pCI372FOR and pCI372REV (Table 4) to verify that mutagenesis had taken place. The purified products were subsequently introduced by electroporation into the strain *L. lactis* NZ9800 which has all the genes necessary for nisin production. Approximately 150 transformants were chosen at random for each single position (P9X and G10X) and 1152 transformants for the randomised P9XG10X bank. Isolated colonies were inoculated into 96-well plates containing GM17 Cm^{10}, incubated overnight and stored at −20°C after addition of 80% glycerol. Deferred antagonism assays were performed by replicating strains on GM17 agar plates and allowing them to grow overnight before overlaying with GM17 agar (0.75%

w/v agar) seeded with the *L. lactis* HP indicator strain. Induction assays were carried out by replicating strains from each 96 well plate into a fresh 96 well plate containing GM17 broth pre-inoculated with *L. lactis* NZ9000 pNZ8150*gfp+*, in which GFP acts as a reporter of expression from a nisin inducible promoter (Field *et al.*, 2019). Induction of GFP was monitored over 20 hours in terms of relative fluorescence units (RFU) using a TECAN Genios Fluorescence, Absorbance and Luminescence Reader using excitation and emission spectra of 485nm and 535nm, respectively.

MALDI TOF Mass Spectrometry

For Colony Mass Spectrometry (CMS), bacterial colonies of P9X and G10X mutants were collected with sterile plastic loops and mixed with 50 μL of 70% IPA containing 0.1% Trifluoroacetic acid (TFA). The suspension was vortexed, the cells centrifuged in a benchtop centrifuge at 8260 *g* for 2 min and the supernatant was removed for analysis. For MALDI TOF Mass Spectrometry of nisin M cell free supernatant (CFS) was purified prepared as follows; a 1% inoculum of nisin mutant producing strains were grown overnight in 50 mL clarified TY broth and incubated overnight at 30°C. Following incubation cells were centrifuged at 5000rpm for 20 mins at 4°C. Cell free supernatant (CFS) was removed and passed through a 1 g (6 mL) Strata C-18 E column (Phenomenex) pre-equilibrated with 6 mL methanol (Fisher Scientific, UK) and 6 mL HPLC grade H_2O. The column was washed with 12 mL 30% ethanol and nisin eluted using 5 mL 70% isopropanol – 0.1% TFA. Mass Spectrometry in all cases was performed with an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5 μL aliquot of matrix solution (alpha-cyano-4-hydroxycinnamic acid (CHCA), 10 mg mL^{-1} in 50% acetonitrile-0.1% (v/v) TFA) was placed onto the target and left for 1-2 min before being removed. The residual solution was then air dried and the sample solution (re-suspended lyophilised powder or CMS

supernatant) was positioned onto the pre-coated sample spot. Matrix solution (0.5 μL) was added to the sample and allowed to air-dry. The sample was subsequently analysed in a positive-ion linear mode.

Purification of nisin A and nisin M

Purifications of nisin A and variant, nisin M were carried out as per a previously employed (Smith *et al.*, 2016) with modifications. Briefly, overnight cultures of *L. lactis* NZ9800 pDF05*nisM* (APC 3920) and *L. lactis* NZ9700 were inoculated at 0.5% into separate purified tryptone-yeast extract (TY) broth (2 × 900 mL) supplemented with 20% glucose and 20% β-glycerophosphate and incubated at 30°C overnight. Following incubation, the cultures were centrifuged at 6500*g* at 4°C for 15 min. The supernatant was passed through a column containing ~70g Amberlite XAD-16 beads and subsequently washed with 500 mL of 30% ethanol. The nisin was eluted from the column using 70% isopropanol containing 0.1% trifluoroacetic acid (TFA). Simultaneously, bacterial cell pellets were resuspended in 300 mL 70% isopropanol – 0.1% TFA and stirred at room temperature for 3 h. This cell suspension was then centrifuged at 5000*g* at 4°C for 10 min and the supernatant was retained. The column eluant was pooled with the post-centrifugation supernatant and isopropanol evaporated using a rotary evaporator (BÜCHI Rotavapor R-205, Switzerland). The pH of the sample was adjusted to pH 4.0 and was subsequently passed through a 10 g (60 mL) Strata C-18 E column (Phenomenex) pre-equilibrated with 60 mL methanol (Fisher Scientific, UK) and 60 mL HPLC grade H_2O. After applying 120 mL 30% ethanol, nisin was eluted from the column using 60 ml 70% isopropanol – 0.1% TFA. For HPLC purification 12 mL volumes were concentrated to a volume of 2 mL by rotary evaporation and applied to a Phenomenex C12 reverse-phase (RP-HPLC) column (Jupiter 4 μm proteo 90 Å, 250 mm × 10.0 mm, 4 μm) previously equilibrated with 25% acetonitrile-0.1% TFA. Nisin was eluted via a gradient

of 25–50% acetonitrile-0.1% TFA that was developed from 10–40 min at a flow rate of 3.2 mL min^{-1}. Nisin containing fractions were pooled and acetonitrile removed by rotary evaporation. The purified peptides were lyophilised and stored at -20°C.

Minimum Inhibitory Concentration (MIC) Assays

MICs were also carried out on strains into which the NICE system was reported to have been introduced including, *Lactobacillus plantarum* (*Lb. plantarum*) and *Lactobacillus brevis* (*Lb. brevis*) in order to determine the potential of nisin M as an alternative to nisin A in the NICE system.

Minimum inhibitory concentration determinations for strains were carried out in triplicate in 96 well microtitre plates (Sarstedt) as described previously (Field, Begley, Paula M O'Connor, *et al.*, 2012). Plates were pre-treated with bovine serum albumin (BSA) prior to addition of the peptides. Briefly, to each well of the microtitre plate 200 µL of phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) was added and incubated at 37°C for 30 min. The wells were washed with 200 µL PBS and allowed to dry. Target strains, *L. lactis* spp. cremoris HP, *L. lactis* NZ9000 pNZ8150*gfp*+ were grown overnight in M17 broth (Sigma) supplemented with glucose (0.5%) at 30°C. *Lb. plantarum* and *Lb. brevis* were grown overnight in MRS broth (Oxoid) at 30°C. Strains were sub-cultured into fresh broth and allowed to grow to an OD_{600} of ~0.5, diluted to a final concentration of 10^5 cfu mL^{-1} in a volume of 0.2 mL. Nisin A and nisin M peptides were adjusted to a 750 nM starting concentration and 2-fold serial dilutions of each peptide was added to the target strain. After incubation for 16 h at 30°C the MIC was read as the lowest peptide concentration causing inhibition of visible growth.

Comparison of nisin A and nisin M induction capacity using beta-galactosidase activity

β-galactosidase activity assay was performed as previously employed (Israelsen *et al.*, 1995) with modifications. Cultures of *L. lactis* NZ9000 pPTPL*βgal*+ were inoculated in M17 broth (Sigma), supplemented with glucose at 0.5% (GM17) and tetracycline (10 µg mL^{-1}), and incubated at 30˚C overnight. Following incubation, a 1% inoculum of each replicate was sub-cultured into fresh GM17 medium and incubated at 30˚C until an OD_{600} of 0.2-0.3 was reached. Cells were then treated separately with nisin A and nisin M purified peptides to a final concentration of 10 ng mL^{-1}. Every hour 1 mL samples of each test were transferred to an eppendorf and centrifuged at 13,000 rpm for 2 minutes (Sorvall Legend Micro 17 centrifuge, Thermo Scientific) to harvest cells. Cells were re-suspended in 1 mL lacZ buffer and 0.5 mL of this was treated with 12.5 µL of 0.1% SDS and 25 µL of chloroform and incubated at 30°C for 5 minutes to dissolve cell membranes. Following incubation 100 µL of 2-Nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg mL^{-1}) (Sigma-Aldrich) was added to each sample and incubated at 37 °C until a yellow colour developed. To stop the reaction samples were treated with 250 µL of a 1 M sodium carbonate solution and centrifuged at 8000 rpm for 5 minutes (Thermo Scientific). Absorbance readings of supernatant were read at OD_{420} and OD_{550} (SpectraMax M3 spectrophotometer, Molecular Devices, Sunnyvale, California, USA). Measurement of β-galactosidase activity of samples was calculated as $1000 \times (OD_{420} - [1.75 \times OD_{550}])/ (t \times v \times OD_{600})$ as previously described (Ge *et al.*, 2016).

Assessment of purified nisin A and nisin M induction capacity using a green fluorescent protein reporter system

Induction assays were performed previously described (Field *et al.*, 2019) with modifications. Briefly, cultures of *L. lactis* NZ9000 pNZ8150*gfp*+ were inoculated in M17 broth (Sigma), supplemented with glucose at 0.5% (GM17) with chloramphenicol (10 µg mL^{-1}) and

incubated at 30˚C overnight. Following incubation, a 1% inoculum of each replicate was sub-cultured into fresh GM17 medium and incubated at 30˚C until an OD_{600} of ~0.5 was reached. Cells were then diluted to a final concentration of 10^5 cfu mL^{-1} and treated with nisin A and nisin M at final concentrations of 10 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1} and 300 ng mL^{-1}. Subsequently, 2 mL was transferred to black, 24 well microtitre plates (PerkinElmer) for induction and 200 µL into a 96 well plate (Sarstedt) for absorbance readings. Fluorescence was detected using a SpectraMax M3 spectrophotometer (Molecular Devices, Sunnyvale, California, USA) where excitation and emission parameters were set to 485nm and 528nm respectively for fluorescence, while absorbance readings were taken at OD_{595} using a Multiskan FC microplate photometer v1.01.14 (Thermo Scientific, Waltham, Massachusetts, USA). Baseline absorbance of un-cultured GM17 was subtracted from the fluorescence and absorbance readings of all test samples using SoftMax Pro v6.3 and SkanIt RE v4.1 software, respectively. Fluorescence was reported as relative light units (RLU) and absorbance as OD_{595nm}. Tests were carried out in triplicate.

Statistical analysis

Statistical analysis was carried out with SPSS Statistics v2. A test of normality was performed to determine data for each test was normally distributed. For normally distributed data a Repeated Measures ANOVA was performed. For data not normally distributed a Levene's test of homogeneity was performed, where if equal variances were assumed the Repeated Measures ANOVA was carried out; and if equal variances were not assumed the non-parametric Friedman test was performed to determine if differences between the two nisin variants induction capacity, and between the growth of the strains when induced with the peptides at higher concentrations compared to an un-induced control were significant. For ANOVA/Friedman's results with a significant difference between groups (P <.05) a post hoc

test was performed. Post hoc tests for normally distributed/equal variances assumed samples was the Bonferroni test, and for non-normally distributed/equal variances not assumed samples Dunnett's T3 test was performed. The significance threshold for all ANOVA's and non-parametric tests performed was set at .05.

Results

Creation and screening nisin derivatives for antimicrobial activity and induction capacity.

Previous studies utilising site-directed and alanine scanning mutagenesis of nisin have revealed that the N-terminal ring structures are an important region required to activate NisRK (Rink *et al.*, 2007; Ge *et al.*, 2016). In particular, mutagenesis of ring B has been shown to modulate antimicrobial and induction activity. We selected this location as a suitable target for the generation of variants to screen for our desired activities. In order to fully exploit the potential of the nisin ring B we undertook a complete randomisation of the two amino acids in Pro9 and Gly10, both alone (P9X, G10X) and in combination (P9XG10X) using NNK scanning of both codons in the nisin A structural gene (*nisA*) as previously described (Field *et al.*, 2008). A bank consisting of 1,452 individual variants created in *L. lactis* NZ9800 pCI372*nisA* (pDF05) were screened for antimicrobial activity using deferred antagonism agar diffusion assays and their ability to induce the nisin promoter, P*nisA* fused to a *gfp* reporter gene. The impact of mutations targeting position nine (proline) on antimicrobial activity was assessed using an overlay assay and resulted in zones ranging from those comparable to the wild type control to those devoid of any observable activity. Analysis of colonies using mass spectrometry and/or DNA sequencing identified 12 different amino acid substitutions corresponding to P9H, P9E, P9S, P9T, P9N, P9A, P9M, P9I, P9V P9L, P9W, and P9F (Figure 1A). Substituting P9 with an alanine (P9A) had no impact on either antimicrobial activity or induction capacity. A number of variants (P9H, P9E, P9W and P9F) displayed a loss of both properties. Several others (P9M, P9L, P9N, P9V and P9I) displayed a significant reduction in antimicrobial activity (between 50-65% of wild type) as well as a reduced ability to induce the *gfp* reporter. Notably, P9T and P9S exhibited a slight reduction in antimicrobial activity (70-75%) but retained 100% and 75% induction capacity

respectively compared to the wild type control (Figure 1A), which was in agreement with previous studies (Rink *et al.*, 2007; Ge *et al.*, 2016). It is significant that replacement of P9 with either threonine or serine introduces hydroxylated residues which could act as substrates for the lanthionine modification machinery. Indeed, colony mass spectrometry (CMS) of the P9T and P9S producers revealed the presence of masses corresponding to the presence of both unmodified (threonine or serine) and modified residues (dehydrobutyrine (Dhb) or dehydroalanine (Dha) (Table1).

Analysis of 144 variants where position 10 (glycine) was targeted, revealed that the majority of clones exhibited either wild type activity or displayed a complete loss of both antimicrobial activity and induction capacity. Mass Spectrometry (MS) and DNA sequencing determined that almost all of the active variants had retained the original glycine at position 10 (wild type), but we also detected variants corresponding to G10T and G10S (Figure 1B). Here too, CMS identified a mixture of both modified and non-modified residues in the case of G10S (i.e. G10S and G10Dha) but this was not observed when threonine was present at position 10. A variant that displayed little reduction in activity (>50%) had an alanine (G10A) substitution (Figure 1B). A selection of variants that lacked both antimicrobial activity and induction capacity were subjected to DNA sequencing analysis, which identified substitutions corresponding to G10F, G10W and G10L. The inability to detect as wide a range of active variants at this position may arise from the fact that several variants in this position (including G10D, G10N, G10H, G10R, G10L and G10P) have been linked with the loss of threonine dehydration at position 8, meaning that ring B does not undergo cyclization (Rink *et al.*, 2007).

We then set out to vary both residues 9 and 10 simultaneously. As expected, screening of the doubly randomized P9XG10X bank revealed far fewer bioactive variants (approx. 5.6% of the total) of which the majority were wild type (38/64). The remainder exhibited varying

degrees of antimicrobial activity ranging from 10-50% with a concomitant loss in induction capacity (data not shown). However, one clone was conspicuous in that despite its apparent lack of antimicrobial activity, it retained an induction capability comparable to the wild type nisin A (Figure 2A). DNA sequencing analysis revealed a variant corresponding to P9T/G10T (both residues replaced with a threonine, Fig 2B). Furthermore, CMS revealed the presence of masses corresponding to the doubly-modified TT peptide (Dhb9Dhb10) but also species with one modified residue to Dhb and a mass close to a peptide with no modified threonine residues (Figure 2A; Table 1). Purification of the derivative P9T/G10T, we termed nisin M, was carried out with our standard nisin purification protocol and subsequent high-performance liquid chromatography (HPLC) evaluation revealed the presence of two major peaks (data not shown). Mass spectrometry analysis of these fractions revealed the presence of one peptide of 3365 Da (consistent with the presence of two Dhb's) and a second peptide of 3383 Da (consistent with a peptide with one threonine and one Dhb). Additionally, a mass in close agreement to a non-modified peptide with threonines in both positions was also observed (data not shown).

Minimum inhibitory concentration (MIC) of nisin M

Following HPLC and freeze-drying of combined fractions to obtain pure peptides, MIC assays were carried out using equimolar concentrations of nisin A and nisin M against a range of Gram positive targets including genera into which the NICE system has been previously introduced (Table 2). The MIC was determined to be the lowest concentration of peptide that resulted in the absence of visible growth of the target strain after 16 hours under the appropriate growth conditions. We established that the MIC of nisin M against a standard laboratory indicator *L. lactis* HP was 2.5 μg mL^{-1}, reflecting a 16-fold increase in MIC compared to wild type nisin A (0.156 μg mL^{-1}). Nisin M displayed a similar decrease in

potency against the *L. lactis* NZ9000 *gfp* reporter strain and its isogenic equivalent *L. lactis* NZ9000 (Table 2). Several lactobacilli have been used as hosts of the NICE system including *Lactobacillus plantarum*, *Lactobacillus helveticus* and *Lactobacillus brevis* (Kleerebezem *et al.*, 1997; Pavan *et al.*, 2000). When *Lb. plantarum* UCC16 and *Lb. brevis* SA-C12 were assessed, an MIC of >2.5 µg mL^{-1} and 1.25 µg mL^{-1} was observed, demonstrating a >4-fold and 16-fold decrease in antimicrobial activity for nisin M respectively in comparison to wild type peptide. (Table 2).

Induction capacity of nisin A and nisin M at 10 ng mL^{-1}

Determination of the induction capacity of nisin A and nisin M at a concentration of 10 ng mL^{-1} was performed using two reporter systems, by way of measurement of GFP and β-galactosidase production. There was no statistical difference in the dynamics of RLU detection when the GFP reporter strain was induced with nisin A and nisin M (P >.05) (Figure 3A). Similarly, induction of the *βgal*+ reporter strain also revealed no significant difference between nisin A and nisin M at equivalent concentrations (10 ng mL^{-1}) (P >.05) (Figure 3B). Moreover, the rate of expression, and therefore the rate of induction was identical for both nisin A and nisin M under the conditions tested in both GFP and β-galactosidase assays.

Induction capacity and effect on growth of nisin reporter strain at higher induction concentrations

Next, we employed 50 ng mL^{-1}, 100 ng mL^{-1} and 300 ng mL^{-1} of peptide to determine the effect of higher concentrations of nisin A and M on both protein expression and on growth of the expression host. Fluorescence (RLU) was measured to determine GFP expression and

absorbance readings at OD_{595nm} were taken to observe growth of induced strains. For each of these higher concentrations a significant difference between the level of induction by nisin A and nisin M (P <.0005) was noted (Fig. 4A-C). Interestingly, the highest RLU reading attained was from cells induced with nisin M at a final concentration of 50 ng mL^{-1}. At this concentration induction continued for the course of the experiment (18 hrs), whereas at 100 ng mL^{-1} and 300 ng mL^{-1} induction reached maximum and decreased after 10-12 hours. Notably, there was no delay in the rate of GFP expression following induction with nisin M at 50 ng mL^{-1} and 100 ng mL^{-1} (Figure 4A-B) compared to 10 ng mL^{-1} (Figure 3A), where fluorescence intensifies at approximately 6 hours post induction for all experiments; meanwhile, there was a minor delay of 30 minutes in expression with induction at 300 ng mL^{-1} (Figure 4C).

When the effects on growth were analysed, no significant impact on the growth of the GFP reporter strain was observed following induction with nisin M at 50 ng mL^{-1} (P> .05), 100 ng mL^{-1} (P> .05) and 300 ng mL^{-1} (P> .05). The OD_{595} of both non-induced cells and cells induced with nisin M increased approximately 5 hours post induction. However, induction with nisin A at the same concentrations resulted in a significant lag-time in growth. An increase in OD_{595} was not observed until 7.5, 8 and 10 hours post induction at 50 ng mL^{-1} (P< .05 for comparison of nisin M to WT, P< .0005 for comparison of WT to uninduced samples), 100 ng mL^{-1} (P< .0005) and 300 ng mL^{-1} (P< .0005), respectively. It is worth noting that although growth was observed by samples at these times following induction with WT nisin, there was no fluorescence detected from the same samples until 12, 16 and 18 hours, respectively (Figure 4A-C & 5A-C).

Discussion

Any new technological advancements to improve the production of protein biopharmaceuticals and industrial enzymes by microorganisms is highly desirable. Potential methods to optimize the efficiency of an inducible gene expression system may involve adjustment of inducer dosage and/or the timing of inducer addition. The Gram-positive NICE system is somewhat unusual in that the inducer peptide also has the capacity to kill the expression host, and thus induction and killing capacity must be balanced. The generation of a nisin derivative that retained its induction properties but with reduced antimicrobial properties would represent a significant improvement to the NICE system that could be applied to more sensitive strains.

Previous work, where the focus has been on the nisin peptide itself, involved randomised mutagenesis of rings A and B (Rink *et al.*, 2007) and described mutants that retained considerable auto-induction abilities but with lower antimicrobial properties (and vice-versa). Similarly, Ge and co-workers (2016) applied a complete alanine scanning mutagenesis approach and reported that the N-terminal ring structures (ring A and ring B) in nisin were involved in activating NisK to act as an inducing molecule (Ge *et al.*, 2016). In this study we focused our attention on ring B with a more systematic mutagenesis approach to identify novel derivatives with altered activity/induction properties. This proved to be successful in that we identified a nisin variant that retains induction capacity that is comparable to the wild type peptide but exhibits significantly less antimicrobial activity.

Notably, another lantibiotic, subtilin is structurally closely related to nisin and contains the same lanthionine ring structure but does not induce P*nisA*. Indeed, in the study by Steiß, Korn, Kötter and Entian (2015) the failure of subtilin to induce the histidine kinase NisK was shown to mostly depend on the presence of an N-terminal tryptophan, as its replacement with the aliphatic amino acid residues isoleucine, leucine, and valine led to activation of NisK

(Spieß *et al.*, 2015). This suggests further bioengineering at position 1 and indeed other amino acid locations in the nisin M background could potentially enhance induction and reduce antimicrobial activity even further.

Although this study highlighted ring B of nisin as a critical region in our quest to separate antimicrobial activity from induction/pheromone activity, more residue positions could and should be targeted. Studies with the natural variant nisin Z have revealed that derivatives corresponding to T2S and M17W exhibited an 11-fold and 2-fold increase in induction capacity relative to the parent peptide, respectively, while derivatives S5T and S3T had significantly reduced induction capacity (Field *et al.*, 2008).

A computational approach evaluating the antimicrobial activity, induction capacity, production levels and immunity/sensor kinase components of natural and bioengineered nisin derivatives could provide a blueprint for the design of more efficient peptide inducers. For example, the ability of nisin P, A and H to activate P*nisA* fused to a *gfp* reporter was assessed and found to differ (Garcia-Gutierrez *et al.*, 2020). The promoter was more sensitive to nisin A (1 ng mL^{-1} – 1 μg mL^{-1}) than nisin H (10 ng mL^{-1} – 1 μg mL^{-1}) and nisin P (100 ng mL^{-1} – 10 μg mL^{-1}). Higher concentrations of nisin P were required to activate the promotor, but it continued to induce promoter activity at higher concentrations (10 μg mL^{-1}) whereas nisin A and H were capable of inducing the promoter only up to 1 μg mL^{-1} concentrations of peptides. The ability to use higher concentrations of nisin P is most likely due to its decreased antimicrobial activity as compared with nisin A and nisin H. While this might advocate for the use of nisin P as an alternative inducer to nisin A, the peptide does not induce at the lower and commonly used inducing concentration (10 ng mL^{-1}). Notably, the nisin M mutant generated in this study induces at both low and high concentrations. While no significant difference in growth of the induced strain compared to the un-induced control was observed, even at the maximum concentration applied (300 ng mL^{-1}), further evaluation with even

higher concentrations of nisin M are necessary and with a variety of expression host strains. However, the practicality of using such high concentrations in terms of industrial applications would need to be considered, given that cell free supernatant from a nisin M producer would be the most likely option for induction (rather than expensive purified nisin peptides); though a fermentate analogous to nisaplin (2.5% nisin A) would enable a range of concentrations to be applied irrespective of the sensitivity of host strains (e.g. induction levels above 10 ng mL^{-1} nisin A results in inhibitory effects on the expression strain *L. lactis* NZ9000) (Kuipers *et al.*, 1998).

Additionally, the natural variant nisin Q also displays similar antimicrobial capabilities to that of nisin A but differs in its ability to induce the *nisA* promoter (Yoneyama *et al.*, 2008). Directed mutagenesis and analysis of the four amino acids which differ between nisin A and nisin Q (A15V, M21L, H27N, I30V) may help us to more completely understand the pheromone activity of nisin. Remarkably, in the study by Ge and co-workers the derivatives L16D, L16A, L16H, L16V, M21A, M21D, and M21N all exhibited enhanced induction properties when assessed by β-Galactosidase assays, with L16D being particularly notable given it also displays a significant reduction in antimicrobial activity (Ge *et al.*, 2016). Other regions of nisin subjected to bioengineering approaches and shown to impact on induction activity include the C-terminus and in particular serine and isoleucine at positions 29 and 30, respectively (Field *et al.*, 2019). Although the specifics of the interaction between the nisin peptide and NisK have yet to be fully elucidated, a recent study has provided some insight through mutational analysis of NisK. Mutagenesis of conserved residues in the extracellular region of NisK revealed that several hydrophobic residues including two aromatic residues (Tyr113 and Phe133) are crucial for NisK in sensing nisin and regulating nisin biosynthesis (Ge *et al.*, 2017).

Elimination of the antimicrobial activity of nisin is a priority when aiming to improve the nisin peptide in terms of its suitability as a peptide inducer, such as in the NICE system. For example, Reunanen & Saris (2003) developed a method for the quantification of nisin in food samples, through the construction of a non-nisin producing *L. lactis* strain (LAC240), with a plasmid containing a *gfp* gene under the control of the *nisF* promoter and the constituent genes of the nisin two-component regulatory system, *nisRK*. It was reported that upon the addition of nisin peptide concentrations greater than 20 ng mL^{-1}, the LAC240 cells became stressed resulting in a reduction in the quantity of GFP produced and the signal reached the background level when the concentration of nisin was approximately 60 ng mL^{-1} (Reunanen and Saris, 2003). Moreover, in a study that aimed to improve the response of *L. lactis* to freezing damage through expression of an antifreeze peptide (SF-P), the recombinant strain *L. lactis* NZ3900 SF-P was incubated with different concentrations of nisin (25, 50, or 100 ng mL^{-1}) and at various pH and growth temperature values (Zhang *et al.*, 2018). Notably, maximal expression was observed at 25 ng mL^{-1}, with a much lower level of expression at 50 ng mL^{-1} and virtually no expression at 100 ng mL^{-1}, most likely due to the inhibitory effects of nisin A, though pH and temperature values were also a factor (Zhang *et al.*, 2018). In another study that sought to optimize the NICE system for the expression of lysostaphin for both laboratory (1 L) and industrial-scale (3000 L) applications and at high cell densities, the authors noted that the addition of too much nisin was detrimental for product formation. Notably, when the culture was induced at higher cell densities, 160 mg L^{-1} lysostaphin was formed with 20 ng mL^{-1} nisin and 220 mg L^{-1} lysostaphin was produced when 40 ng mL^{-1} nisin was used for induction, indicative of a clear correlation between the cell density at induction and the amount of nisin that is needed for maximal induction (Mierau *et al.*, 2005). While this group reported that maximum protein yield in the NICE system is achieved by induction carried out at a cell density of OD_{600} = 5 with a final concentration of 40 ng mL^{-1} of

nisin, we suggest that Nisin M provides for a greater flexibility with respect to inducer concentration by virtue of the attenuated antimicrobial activity of the peptides and the application of high concentrations of inducer peptide is not now a limiting factor.

To date, a multitude of peptides, enzymes and vaccines of clinical and biotechnological interest have been overexpressed using nisin, including the anti-bacterial protein lysostaphin (Mierau *et al.*, 2005), a haemagglutinin of the H5N1 influenza virus (Szczepankowska *et al.*, 2017) and Rotavirus VP6 Protein (Esteban *et al.*, 2013), to name but a few. Though several improvements have been made to the NICE system, further improvements are possible. For example, streamlined-genome mutants of *L. lactis* NZ9000 were generated by deletion of four large nonessential DNA regions accounting for 2.83% of the genome and evaluated as microbial cell factories for recombinant protein production. Indeed, following nisin induction, not only was the transcriptional efficiency improved but also the production levels of the expressed reporters were approximately three to fourfold enhanced compared with the wild strain (Zhu *et al.*, 2017). Additionally, expression from the Δ*lac*F host-strain *L. lactis* NZ3900 (a strain unable to utilize lactose), enabled food-grade, lactose-based plasmid selection and induction (Platteeuw *et al.*, 1996) whilst deletion of a specific proteinase gene (NZ9000 Δ*htrA*) led to increased stability of heterologous-secreted proteins (Lindholm, Smeds and Palva, 2004).

While the aforementioned studies focused on improving the host strain for expression of proteins, this study focuses on potential improvements that can be made to the inducing peptide via mutagenesis of ring B, which has already been reported as playing an important role in induction capacity (Rink *et al.*, 2007; Ge *et al.*, 2016). This study has demonstrated that a nisin A variant with modifications to ring B retained comparable induction capacity to the wild type nisin A peptide yet exhibited less inhibitory effects on the growth of the strain *L. lactis* NZ9000 when applied at concentrations as high as 300 ng mL^{-1} (0.09 μM). It was

also determined this combination has between >4 and >16 fold less activity against various genera and species of bacteria into which the NICE system has been introduced, therefore supporting the claim that nisin M exhibits potential as a suitable alternative to nisin A for use in the NICE system.

This study confirms that random mutagenesis experiments continue to be beneficial with a view to enhancing the functional properties of the nisin peptide for specific applications and provide novel nisin variants that exhibit potential for future applications in the pharmaceutical, biotechnological and industrial fields.

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Tables and Figures

Table 1. Mass spectrometry analysis of selected derivatives

Ring B Derivative	Predicted Mass (Da)	Actual Mass (Da)	Dehydrations		Ref
			Observed	Lacking	
P9A	3328	3327.87	8	0	This study (Ge *et al.*, 2016)
P9T	3357	3356.67	8	1	This study
		3339.62	9	0	
P9S	3343	3342.73	8	1	This study
		3324.69			
G10A	3366	3367.14	8	0	This study (Rink *et al.*, 2007)
G10T	3398	3397.76	8	1	This study (Rink *et al.*, 2007)
G10S	3384	3384.57	8	1	This study
		3367.23	9	0	(Rink *et al.*, 2007)
P9T/G10T (nisin M)	3402	3399.86	8	2	This study
		3382.91	9	1	
		3365.33	10	0	
Nisin A	3354	3353.44	8	0	

Table 2. MIC of nisin A and nisin M against standard indicator strains (including those reported to have had the NICE system introduced).

Indicator organism	Nisin A µg mL^{-1} (µM)	Nisin M µg mL^{-1} (µM)	Fold decrease in activity
Lb. plantarum UCC16	0.625 (0.1875)	>2.5 (>0.750)	>4
L. lactis NZ9000 pNZ8150*gfp*+	0.156 (0.046)	>2.5 (>0.750)	>16
L. lactis NZ9000 pNZ8150	0.156 (0.046)	2.5 (0.750)	16
Lb. brevis SA-C12	0.078 (0.0234)	1.25 (0.375)	16
L. lactis spp. *cremoris* HP	0.156 (0.0468)	2.5 (0.750)	16

Table 3. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Characteristic	Reference
L. lactis NZ9000	MG1363 derivative, *NisRK* integrated into *pepN* gene (*pepN*-). Most commonly used host of the NICE system.	(Kuipers *et al.*, 1998) (Mierau and Kleerebezem, 2005)
L. lactis NZ9000 pNZ8150	NZ9000 strain harbouring pNZ8150. pNZ8150: *Sca*I site for translational fusions, standard vector for NICE system, Cm^R.	(Mierau and Kleerebezem, 2005)
L. lactis NZ9000 pNZ8150*gfp*+	NZ9000 strain harbouring pNZ8150 *gfp*+ under P*nisA* promoter. Cm^R.	(Field *et al.*, 2019)
L. lactis NZ9000 pPTPL*βgal*+	NZ9000 strain harbouring low copy plasmid pPTPL with β-galactosidase expressing gene under the control of the P*nisA* promoter. Tet^R	(Field *et al.*, 2008)
L. lactis NZ9800	Derivative of NZ9700 with 4bp deletion rendering an inactive nisin operon (Δ*nis*A), except *nisRK* genes. Host of the NICE system.	(Oscar P Kuipers *et al.*, 1993; Kuipers *et al.*, 1998)
L. lactis NZ9800 pDF05	NZ9800 harbouring pDF05 (pCI372 with *nisA* under its own promoter). Wild type nisin A producer, Cm^R.	(Oscar P Kuipers *et al.*, 1993; Kuipers *et al.*, 1998) (Field *et al.*, 2008)
L. lactis NZ9800 pDF05*nisM*	pDF05 where codons 9 and 10 of *nis*A have been randomized. Nisin M producer, Cm^R.	This work UCC Culture Collection (APC 3920)
Lb. plantarum UCC16	Nisin sensitive indicator Species in which NICE system has been utilized.	UCC Culture Collection (Pavan *et al.*, 2000; Mierau and Kleerebezem, 2005)

Table 3. continued

Lb. brevis SA-C12	Nisin sensitive indicator Species in which NICE system has been utilized.	UCC Culture Collection (Åvall-Jääskeläinen *et al.*, 2002; Mierau and Kleerebezem, 2005)
L. lactis ssp. *cremoris* HP	Nisin sensitive indicator strain	UCC Culture Collection

Table 4. Oligonucleotides utilised in this study.

Primer name	Sequence
NisP9degFOR	5’ CTA TGT ACA NNK GGT TGT AAA ACA GGA GCT CTG ATG GGT 3’
NisP9degREV	5’ TTT ACA ACC MNN TGT ACA TAG CGA AAT ACT TGT AAT GCG 3’
NisG10degFOR	5’ TGT ACA CCC NNK TGT AAA ACA GGA GCT CTG ATG GGT TGT 3’
NisG10degREV	5’ TGT TTT ACA MNN GGG TGT ACA TAG CGA AAT ACT TGT AAT 3’
NisP9G10degFOR	5’ CTA TGT ACA NNK NNK TGT AAA ACA GGA GCT CTG ATG GGT 3’
NisP9G10degREV	5’ TTT ACA MNN MNN TGT ACA TAG CGA AAT ACT TGT AAT GCG 3’
pCI372For	5’ CGGGAAGCTAGAGTAAGTAG 3'
pCI372Rev	5’ ACCTCTCGGTTATGAGTTAG 3’

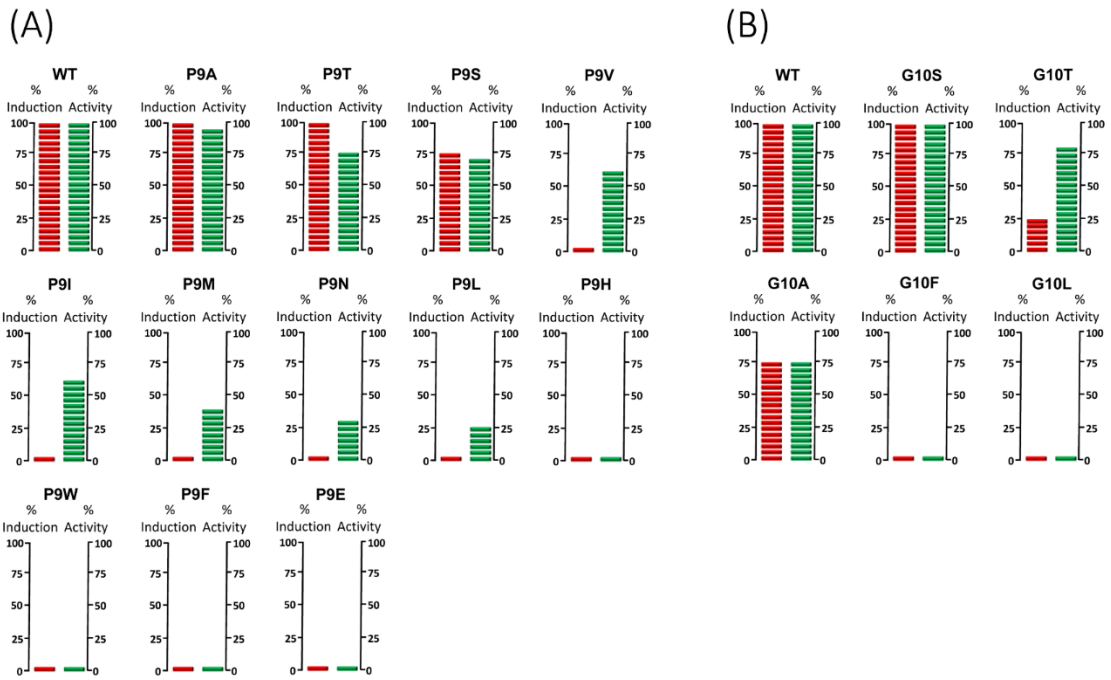


Figure 1. Induction and antimicrobial activity analyses of nisin mutants with substitutions at **(A)** position 9 and **(B)** position 10. Induction capacity (red) and antimicrobial activity (green) is shown as percentages (%) and ordered from highest to lowest based on biological activity of the variants.

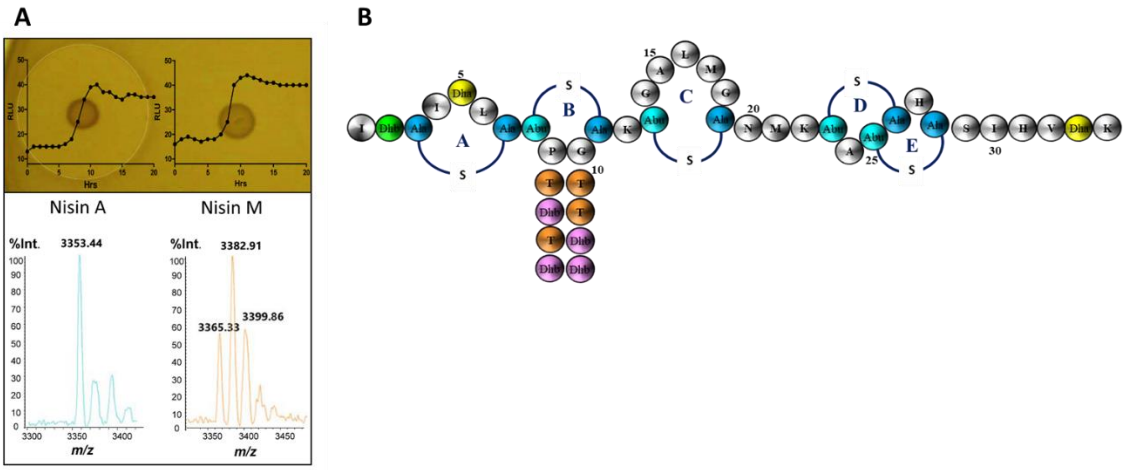


Figure 2. (A) Top- Biological activity of nisin A and nisin M as determined by deferred antagonism assays and assessment of induction capacity following induction of a *L. lactis* strain containing *gfp*+ under control of the nisin promoter. **Bottom-** Colony Mass Spectrometry of the wild type nisin A producer (3353.44 Da) and nisin M comprising of a combination of unmodified peptide, single dehydration or two dehydrations at P9T/G10T (3399.86 Da, 3382.91 Da, and 3365.33 Da respectively). (**B)** Structure of nisin A where amino acids are represented by their single letter codes and modified residues are indicated as follows; Dha: dehydroalanine, Dhb: dehydrobutyrine, Abu: 2-aminoabutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: methyllanthionine. Residues in orange and pink show amino acid substitutions for nisin M, producing 4 possible forms of the peptide.

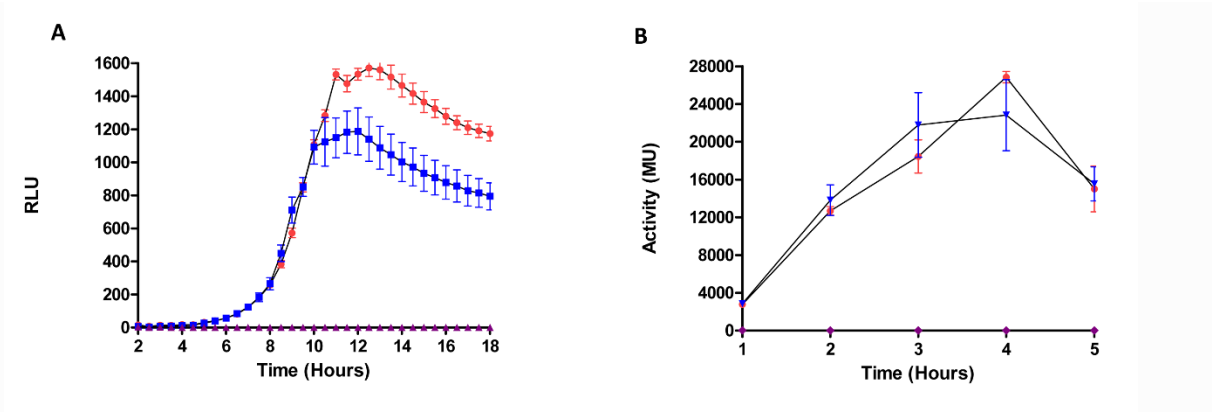


Figure 3. Induction capacity of nisin A (red/circle) and nisin M (blue/square) determined by expression of (A) GFP and (B) β-galactosidase reporter genes under the control of the P*nisA* promoter in *L. lactis* NZ9000 pNZ8150*gfp*+ and *L. lactis* NZ9000 pPTPL*βgal*+ respectively when induced at a final concentration of 10 ng mL^{-1}. Negative controls (green/triangle) are uninduced test strains. Statistical analysis shows there is no significant difference between the induction capacities of nisin M and nisin A in both methods tested (*P*> .05).

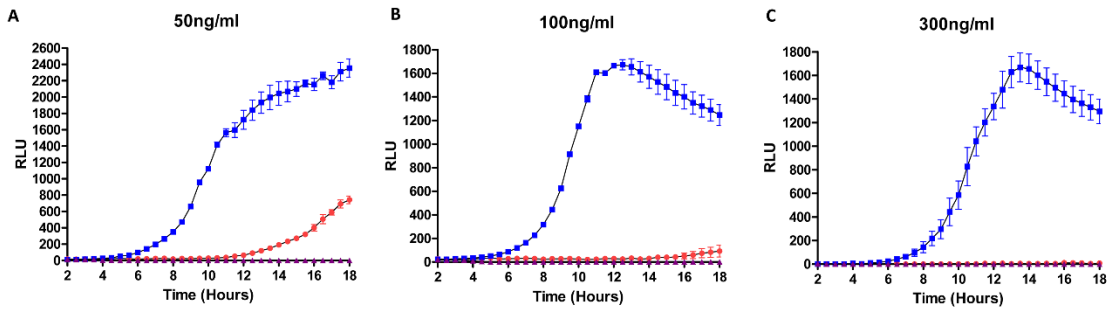


Figure 4. Comparison of induction capacities of nisin A (red/circle), nisin M (blue/square) determined by expression of GFP under the control of the P*nisA* promoter in *L. lactis* pNZ8150*gfp*+ induced at final concentrations of (A) 50 ng mL^{-1}, (B) 100 ng mL^{-1} and (C) 300 ng mL^{-1}. Statistical analysis demonstrates a significant difference between induction capacity of the two peptides at all concentrations tested, ($P < .0005$) Negative control in this assay is uninduced *L. lactis* NZ9000 pNZ815*0gfp*+ (green/triangle).

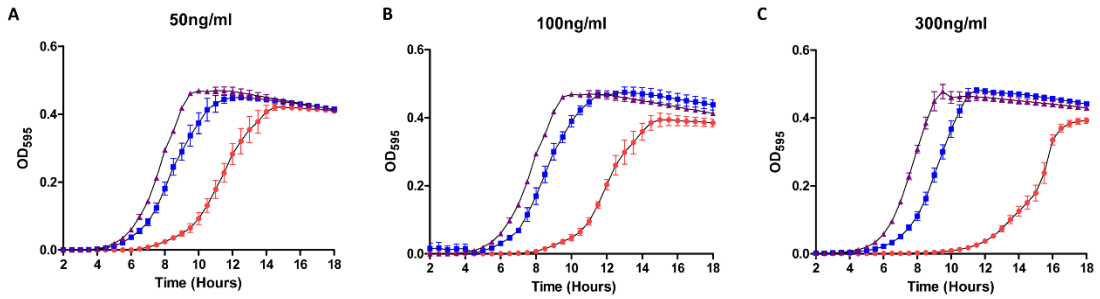


Figure 5. Effects of nisin A (red/circle) and nisin M (blue/square) on growth of *L. lactis* NZ9000 pNZ8150*gfp*+ induced at concentrations of (A) 50 ng mL^{-1}, (B) 100 ng mL^{-1} and (C) 300 ng mL^{-1} compared to an un-induced control (green/triangle) determined by absorbance at OD_{595nm}. Results show no significant difference between growth of the uninduced control and cells induced with nisin M at all concentrations tested ($P > .05$); while there is a significant difference between the growth of cells induced with WT nisin compared to both the uninduced control (50 ng mL^{-1}: $P < .0005$; 100 ng mL^{-1}: $P < .0005$; 300 ng mL^{-1}: $P < .0005$), and samples induced with nisin M (50 ng mL^{-1}: $P < .05$; 100 ng mL^{-1}: $P < .0005$; 300 ng mL^{-1}: $P < .0005$).

Chapter 4. Screening canine sources for novel antimicrobials reveals the circular broad-spectrum bacteriocin, caledonicin produced by *Staphylococcus caledonicus*



Buttons

Abstract

Antimicrobial-resistant pathogens present an ongoing threat to human and animal health, with deaths linked to antimicrobial resistance (AMR) predicted to increase yearly. While the misuse and overuse of antibiotics in humans undoubtedly contribute to this escalation, antibiotic use in the veterinary field, including companion animals, also plays a contributing role. Pet owners' desire to improve the quality of life of their pets is likely to support antibiotic use within this field. Consequently, there is a need for antibiotic alternatives to treat bacterial infections. This study set out to screen for antimicrobial peptides known as bacteriocins from canine sources. Following a laboratory-based protocol, 22 bacterial isolates were subjected to whole genome sequencing (WGS) and a total of 14 putative novel bacteriocins were identified from both class I and II bacteriocin classes, confirming that canines are a rich environment for bacteriocin-producing strains. One particular bacteriocin, herein named caledonicin, was identified via *in silico* analysis from a *Staphylococcus caledonicus* strain, and partially purified for further *in vitro* evaluation. Caledonicin is a 64 amino acid (IAANLGVSSGTAYSMANALNNISNVATALTIIGTFTGVGTIGSGIAATILAILKKKGV AAAAAF) novel circular bacteriocin most closely related to enterocin_NKR-5-3B based on core peptide alignment (39.1%), with a molecular weight of 6077.1 Da. Caledoncin exhibits a broad spectrum of activity against a range of pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), methicilling-resistant *Staphylococcus pseudintermedius* (MRSP), and *Listeria monocytogenes*; and the gut related bacterium associated with Crohn's disease, *Mediterraneibacter gnavus* ATCC 29149 (previously *Ruminococcus gnavus* ATCC 29149). This represents the first bacteriocin screening study involving bacteria from canine sources, and the first novel bacteriocin to be identified and characterised from this staphylococcal species.

Introduction

Antimicrobial resistance (AMR) can be either innate or acquired, where innate resistance is due to bacteria evolving over a long period of time to environmental change, whereas acquired resistance develops over a short period due to selective pressure from exposure to antibiotics (Palma, Tilocca and Roncada, 2020).

While AMR is a serious health concern for humans, it is also a problem in veterinary settings (Meade, Slattery and Garvey, 2020). A contributing factor in the development of resistance in animals is the misuse and/or overuse of antibiotics, including their use for improved growth in livestock. It is reported that over 70% of all antibiotics (including those defined as medically important for treatment of infections in humans by the Food and Drug Administration [FDA]) are also sold for use in animals in the USA (O'Neill, 2019).

Unlike the many reports of the acquisition of antibiotic resistance genes (ARGs) in livestock associated with the use of antibiotics, there is a scarcity of such data in companion animals (Pomba *et al.*, 2017; Rendle and Page, 2018). The spread of antibiotic-resistant bacteria between pets and humans can occur via contact with the animal, physical injuries (Caneschi *et al.*, 2023), or transfer of genetic material from the owner's residential microbiota to that of the pet, or *vice-versa* (Rendle and Page, 2018). Although it appears the use of antibiotics in companion animals has a less significant impact on the development and spread of ARGs compared to that of humans and livestock based on sales data (Graham *et al.*, 2019), this is still a sector of animal health that requires more attention (Caneschi *et al.*, 2023). This includes a proposal for the development of new antimicrobial compounds for the treatment of antibiotic-resistant infections caused by pathogenic bacteria in the veterinary field (World Health Organisation, 2021), including MRSA, MRSP, *Pseudomonas aeruginosa*, extended-spectrum beta-lactamase (ESBL) *Escherichia coli*, *Klebsiella pneumoniae* and vancomycin-resistant *Enterococcus* sp. (VRE) (Palma, Tilocca and Roncada, 2020; Caneschi *et al.*, 2023).

One potential alternative treatment for these infections is bacteriocins, antimicrobial peptides produced by bacteria that target closely related species. Bacteriocins are divided into two classes based on the presence (Class I) or absence (Class II) of post-translational modifications (PTMs) (Soltani *et al.*, 2021). Nisin is a class I lantibiotic and the most studied bacteriocin since its discovery in 1928 and has antibacterial activity against a range of pathogens including veterinary-related organisms such as *Enterococcus faecium*, *Streptococcus agalactiae*, and *Staphylococcus aureus* (Field *et al.*, 2023). There have been a number of nisin-based products formulated for treatment of veterinary-associated infections. These include Ambicin® (Applied Microbiology, Inc., New York, NY), MastOut® and WipeOut® (Immucell Corporation), which have been shown to be effective treatments of bovine mastitis caused by *S. aureus* in cows (Sears *et al*., 1992; Cotter *et al*., 2005; Cao *et al*., 2007). Bayer have also developed nisin-incorporated wipes (Preva® Medicated Wipes) containing 25µg ml^{-1} nisin for topical use in dogs, cats and horses (Field *et al.*, 2015), highlighting the potential for bacteriocins instead of, or in combination with antibiotics for therapeutic use in veterinary medicine.

In this study, we screened bacteria isolated from canine sources for antimicrobial activity with the aim of finding and characterising putative novel bacteriocins that could aid in the fight against AMR. To our knowledge, this is the first such study to search for putative bacteriocins from canine sources. One of 14 putative novel bacteriocins identified in this study via whole genome *in silico* analysis was a circular bacteriocin, similar to enterocin-NKR-5-3B. This bacteriocin was produced by a novel species of *Staphylococcus*, *Staphylococcus caledonicus*, that was first isolated and identified also from a canine in Scotland in 2021 (Newstead *et al.*, 2021). As a consequence of the novelty in terms of bacterial species, its canine-related source and its circular nature, this bacteriocin designated caledonicin was selected for further characterisation. To that end, semi-purified preparations of caledonicin were obtained and

subjected to an array of conventional bacteriocin assessment methods including MALDI TOF mass spectrometry, protease sensitivity, heat stability, as well as spectrum of inhibition assays against a variety of relevant bacterial indicator organisms. This is both the first bacteriocin to be reported and characterised from the *S. caledonicus* species and the first circular bacteriocin from the *Staphylococcus* genus to be confirmed *via* correlation of MALDI TOF mass spectrometry of an active extract which exhibits antimicrobial activity against both veterinary- and human-related pathogens.

Materials and Methods

A schematic representation of methods performed in this study is presented in Figure 1.

Isolation of antimicrobial isolates from canine sources

Five canines (4 females and 1 male), ranging between 7 and 11 years of age at the time of swabbing participated in this study. Four areas of the body were swabbed: the ear canal, the axillary vault (underarm), the gumline/teeth, and the inner nares (nostril). Each swab (Aptaca, sterile swabs; Aptaca S.p.A, Regione Monforte, 30-14053 Canelli, Italy) was dipped in sterile saline solution (PBS) before swabbing the site of interest, and swabs were stored for no more than 2 hr at 4˚C before tenfold serial dilutions were performed in PBS and 100 μL aliquots were spread-plated onto Brain Heart Infusion (BHI) agar (Merck, Darmstadt, Germany) and Tryptic Soy Agar (TSA) (Merck Millipore)**.** All plates were incubated aerobically at 37˚C for 24-48 hr.

Detection of antimicrobial activity from bacterial isolates from canines

Deferred antagonism assays were performed for detection of antimicrobial production by bacterial isolates from canine sources as follows. A 1% inoculum of indicator organism *Lactococcus lactis* subsp. *cremoris* HP (*L. lactis* HP) was sub-cultured overnight in 10 mL of M17 broth supplemented with glucose (GM17) (0.5% w/v) and incubated at 30˚C overnight. Plates with bacterial isolates from canine samples grown as described above on BHI and TSA were overlaid with GM17 sloppy agar (0.75% w/v agar) seeded with 0.25% inoculum of *L. lactis* HP indicator strain and grown aerobically overnight at 30˚C (see Table 1 for optimal growth conditions of bacterial strains used in this study). Colonies from this assay that

exhibited zones of inhibition were then inoculated into BHI broth, grown overnight at 37˚C and stocked in 40% glycerol at −80˚C for further characterisation.

Characterisation of antimicrobial-producing canine isolates

Cross Immunity

To determine the relatedness of bacteriocins produced by the antimicrobial-producing isolates, cross-immunity assays were performed using deferred antagonism assays as previously described. Briefly, 96-well microtitre plates with all antimicrobial-producing isolates were stamped (approximately 2 µL) onto BHI agar plates and incubated overnight at 37˚C. The spots were then subjected to UV treatment in a CL-1000 Ultraviolet Crosslinker for 45 min. Following this, 20 mL volumes of sloppy BHI (at 0.75% w/v agar), were prepared and inoculated individually with 0.25% of an overnight culture of each antimicrobial-producing canine isolate. Plates were overlaid with the inoculated sloppy-agar and incubated overnight at 37˚C.

16S rRNA sequencing

Colony PCR was performed on antimicrobial-producing strains. Cells were lysed in 25 µL of PCR-grade water via microwaving for 50 seconds and centrifuged at 11,500 xg for 1 min. PCR was performed in a total volume of 50 µL using 25 µL of MyTaq™ Mix PCR master mix (Bioline), 19 µL of PCR-grade water, 2 µL of the non-specific primers 27F (5' – AGAGTTTGATCATGGCTCA – 3') and 1492R (5' – TACGGTTACCTTGTTACGACTT – 3') (primer stocks at 10 µM; Eurofin Genomics) and 2 µL of DNA template from lysed cells. Amplification was carried out with reaction conditions as follows: initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, annealing at 55°C for 15 s and elongation at 72°C for 10 s with a final extension step at 72°C for 10 min. Five µL of the resulting amplicons

from each reaction were electrophoresed in a 1.5% (w/v) agarose gel. An OmegaFlour Plus ™ Gel Documentation System (Aplegen, San Francisco, United States) was used for visualization. The PCR products were purified using the GeneJet PCR Purification Kit (Thermo Fischer Scientific, Waltham, MA, United States). DNA sequencing of the forward strand was performed by Genewiz (Leipzig, Germany). Resulting sequences were identified to species level (≥98%) using BLAST for comparison to sequences deposited in the GenBank database.

Whole Genome Sequencing (WGS) and in silico *mining for bacteriocin operons*

DNA from 22 antimicrobial-producing isolates selected for whole genome sequencing was extracted by growing cultures overnight in 10 mL BHI broth at 37°C. Genomic DNA was extracted using a Sigma-Aldrich DNA purification kit as described by the manufacturer (Sigma-Aldrich Ireland Limited, Vale Road, Arklow, Co. Wicklow, Ireland). A total of 22 genomes were subjected to WGS, of which 12 (APC 4160, 4164, 4166, 4149, 4130,4133, 4148, 4170, 4140, 4157, 4158, & 4171) were sequenced with Illumina MiSeq Sequencing System by GenProBio (University of Parma). The remaining genomes (APC 4161, 4163, 4136, 4156, 4152, 4153, 4147, 4137, 4145 & 4154) were sequenced via the Illumina sequencing platform with MicrobesNG (Birmingham, UK). WGS was downloaded in FASTQ format. Quality control was run on the reads using FastQC v0.11.9 and Fastp v0.23.2. The genomes were assembled using Spades v3.15.5. Quality control was run on the genomes using CheckM2 v1.01. Genome assemblies were annotated using Bakta v1.8.1 and database v5. *In silico* bacteriocin mining tools BAGEL4 (Van Heel *et al.*, 2018) and antiSMASH7.0 (Blin *et al.*, 2023), were used for analysis of genome sequences to identify putative novel bacteriocin operons. To determine the level of novelty of bacteriocins identified, and to compare these operons to existing bacteriocins against the National Centre for Biotechnology Information (NCBI) database, the BLASTP (Protein Basic Local Search Tool) tool was used. Amino acid

sequences of putative novel bacteriocins predicted in whole-genome sequenced isolates were aligned with closely related bacteriocin leader and core peptides using the Multiple Sequence Alignment (MSA) tool MUSCLE accessed via EMBL's job dispatcher (Madeira *et al.*, 2022) and then visualized using Jalview (Waterhouse *et al.*, 2009); where pairwise alignment was performed, and percentage identity calculated to determine most closely related known bacteriocin to the ones found in this study. It should be noted that MSA was only carried out on circular bacteriocin, lasso peptide and lanthipeptide classes of bacteriocins which are the main focus of our research group. Although novel bacteriocin operons belonging to the thiopeptide/thiazole/oxazole-modified microcins (TOMMs) class were identified in the course of this study, they were not examined in detail but may be the subject of more focused investigation in future studies. Bacteriocins were considered novel where one or more amino acid changes in their core peptide were observed compared to their most closely related bacteriocin.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was performed on colonies from the twenty-two antimicrobial-producing isolates subjected to whole genome sequencing and partially purified 70% IPA fraction of caledonicin. Briefly, colonies were mixed with 50 µL propan-2-ol 0.1% TFA, vortexed three times and centrifuged at 16 000 × *g* for 30 s. MALDI TOF mass spectrometry was performed on the cell-free supernatant (CFS) using an iDPlus Performance MALDI TOF mass spectrometer (Shimadzu Europa GmbH , Duisberg, Germany). An aliquot (0.5 µL) of matrix solution (α-cyano 4-hydroxy cinnamic acid, 10 mg mL^{-1} in acetonitrile-0.1% (v/v) trifluoroacetic acid) was deposited onto the target and left for 20 s before being removed. The residual solution was allowed to air-dry and 0.5 µL of the sample solution was deposited onto the pre-coated sample spot; 0.5 µL of matrix solution was

added to the deposited sample and allowed to air-dry. Samples were then analysed in positive-ion linear mode.

Spectrum of inhibition of putative novel bacteriocin producers

Deferred antagonism assays to determine the spectrum of inhibition of the eight isolates harbouring putative novel bacteriocin operons were conducted as previously described, with minor modifications. Approximately 2 µl of an overnight culture was spotted on BHI agar (1.5%) plates via a 96-well plate replicator (Boekel, Germany) before incubating aerobically at 37 °C overnight. Following incubation, spots were chloroform-treated for 30 min before overlaying with eight gram-positive and seven gram-negative indicator strains as described above. Indicator strains employed to detect antimicrobial activity and their growth conditions are listed in Table 1A. Zones of inhibition, indicative of antimicrobial activity, were measured using Vernier calipers, (resolution 0.05), recorded in millimetres (mm) and rounded to one decimal place. Assays were performed in duplicate. Activity was calculated as follows: diameter of zone minus diameter of colony in millimetres and expressed as the average between two.

Characterisation of a novel circular bacteriocin, caledonicin, from *S. caledonicus* APC4137

Assessement of bacteriocin production in S. caledonicus APC4137 extracts

S. caledonicus APC 4137 extracts including CFS, a 70% IPA 0.1% TFA cell extract and freeze-thaw liquid (FT) from agar on which the strain was grown were tested against *L. lactis* HP and *Micrococcus luteus* (*M. luteus*) APC4061 for activity via WDA. Briefly, M17 supplemented with 0.5% glucose and BHI agar was seeded with a 1% incoculum of indicators

L. lactis HP and *M. luteus* APC 4061, respectively. Wells were bored into the agar plates and 50 µL of each extracts was inoculated into wells followed by incubation overnight at temperatures relevant to the indicators tested (Table 1). Following incubation wells were checked for zones of inhibition.

Partial purification of caledonicin from S. caledonicus APC 4137

Freeze-thaw liquid for semi purification of caledonicin was prepared as follows. *S. caledonicus* APC 4137 was grown overnight in BHI at 37 °C and 500 µL of this overnight culture was spread plated on BHI sloppy agar (0.75%) plates before incubating overnight at 37 °C. Following incubation, the plates were then overlaid with GM17 sloppy agar, inoculated with a 0.25% inoculum of *L. lactis* HP before incubating overnight at 30 °C. Following incubation and observation of antimicrobial activity of the indicator overlay, the plates were frozen at -80 °C for 1 hour, followed by thawing at room temperature. FT liquid from thawed plates was collected, filter sterilised through a 0.2 µm filter and subsequently passed through a 10-g (60 mL) Strata C18-E SPE column (Phenomenex) pre-equilibrated with 60 mL methanol (Fisher Scientific, UK) and 60 mL HPLC-grade H_2O. After 60 mL of 30% ethanol was applied, the antimicrobial was eluted from the column using 60 mL of 50%, 60% and 70% isopropanol. Eluted fractions were tested for antimicrobial activity against *L. lactis* HP via WDA for activity. Samples were also subjected to MALDI TOF mass spectrometry (described below) to determine the presence of the corresponding mass of the circular bacteriocin.

Heat stability and protease sensitivity of caledonicin from S. caledonicus APC 4137.

The heat stability and protease sensitivity of caledonicin were determined by WDA on the semi-purified C18 fraction eluted in 70% IPA, following confirmation of the predicted mass by MALDI TOF mass spectrometry. The C18 70% eluent was incubated for 15 min at a range

of temperatures, 37˚C, 65˚C, 80˚C, 100˚C, and 120˚C, before performing a well-diffusion assay (WDA) in 1.5% M17 agar supplemented with 0.5% glucose seeded with 0.25% of *L. lactis* HP. Untreated C18 70% eluent was used as a control. To investigate if the antimicrobial produced was proteinaceous in nature, the eluent was subjected to protease treatment with Proteinase K (Sigma) to a final concentration of 20 mg mL^{-1} at 37˚C for 3 hr before heat deactivating the protease enzyme at 95˚C for 5 mins and testing for antimicrobial activity via WDA as described above. Controls for this test included untreated caledonicin containing eluent (diluted to the same concentration as test caledonicin containing eluent) and heat-treated caledonicin containing eluent (95˚C for 5 mins) without protease treatment. To determine the specific protease sensitivity of caledonicin the semi-purified fraction was treated with trypsin, α-chymotrypsin, pepsin and proteinase K (Sigma) to a final concentration of 100 µg mL^{-1} and incubated at 37˚C for 3 hrs. Enzymes were deactivated with heat treatment as above before testing for antimicrobial activity against *L. lactis* HP via WDA.

Antimicrobial activity spectrum of caledonicin

Following confirmation of activity of the semi-purified caledonicin preparation against L. lactis HP and the presence of the correct predicted molecular mass, the spectrum of inhibition of caledonicin was determined via WDA against the eight gram-positive indicators used in overlay assays for all novel bacteriocin producers in this study (Table 1A); and six other gram positive indicators. Four of these six partially make up a Simplified Human Intestinal Microbiota (SIHUMI) consortium (Ríos Colombo *et al.*, 2023a). This simplified gut consortium included *Enterococcus faecalis* OG1RF, *Lactiplantibacillus plantarum* WCFS1, *Bifidobacterium longum* ATCC 15707 and *Mediterraneibacter gnavus* ATCC 29149 with the addition of *Clostridioides difficile* APC43 indicator (Table 1B). These strains were grown at 37 °C under strict anaerobic conditions (Type A vinyl anaerobic chamber, Coy Labs) in a

modified Brain Heart Infusion (Oxoid) medium with the addition of 0.5% yeast extract, 5 mg/L hemin (Sigma–Aldrich), 1 mg/ml cellobiose (Sigma–Aldrich), 1 mg/ml maltose (Sigma–Aldrich), and 0.5 mg/ml L-cysteine (Sigma–Aldrich) – LYHBHI. *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901 was also used as an indicator in this assay, and grown anaerobically in MRS media at 37 °C (Table 1B).with the addition of *Clostridioides difficile* APC 43 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901 indicators (Table 1B).

Structural prediction of caledonicin

Structural characterisation of the circular bacteriocin from *S. caledonicus* APC4137 was performed via the online tool ColabFold v1.5.5: AlphaFold2, followed by visualisation of the bacteriocin in the PyMOL Molecular Graphics System, v 3.0 (Schrödinger, LLC) with an extension prompt to colour structures from the AlphaFold Protein Structure Database by predicted local distance difference test (pLDDT) where dark blue regions indicate high confidence (pLDDT > 90).

Results

Cross-immunity of antimicrobial-producing bacterial isolates from canine:

Based on the presence of zones of inhibition against the indicator *L. lactis* HP in agar overlay assays, 42 antimicrobial producers were isolated from approximately 5000 colonies screened from the five canines in this study. Cross-immunity tests were performed for isolates from each dog against each other isolate. All producers were immune to the activity of their own antimicrobial(s), with the exception of four strains (APC 4132, APC 4133 and APC 4150 from canine 1 and APC 4160 from canine 3) (Figure 2 and Supplementary Figure S1). Isolates selected for further testing were based on this assay. Isolates APC 4130-4134 and APC 4148-4150 from canine 1 all exhibit a similar trend in activity, indicating the probability that these isolates are the same strain or are producing the same antimicrobial (Figure 2). This was supported by 16S rRNA sequencing to species level where all of these isolates were identified as *Bacillus cereus*. Isolates APC 4137 and APC 4140 exhibit completely different activity profiles compared to all other isolates from that same canine and were therefore selected for further characterisation. Isolates APC 4137 and APC 4140 were confirmed by whole genome sequencing to be *S. caledonicus* and *Bacillus pumilus*, respectively. Although some isolates, such as *B. cereus* APC 4147 and APC 4145, exhibited little activity against indicators in this assay, they did inhibit growth of the indicator *L. lactis* HP in the original overlay assays. Therefore, the isolates brought forward for further analysis were those determined by the results of both cross-immunity and those with activity in the initial deferred antagonism assays against *L. lactis* HP. These methods of initial characterisation and the basis for bringing these specific isolates of interest forward in this study could be considered a limitation due to the use of only one indicator in the initial screening process, as well as the limited number of media used for bacterial growth. Cross-immunity was also performed for the isolates from all other canines in this study (Figure S1).

16s rRNA sequencing

16s rRNA sequencing of antimicrobial-producing bacterial isolates revealed a total of eight different species across the 42 isolates sequenced. The most abundant genus isolated in this study was *Staphylococcus* (n=24), followed by *Bacillus* (n=15), *Actinomyces* (n=2) and *Paenibacillus* (n=1).

Bacteriocin identification via in silico *mining of whole genomes and AMR/virulence genes detection*

The 22 genomes subjected to whole genome sequencing represent a range of antimicrobial-producing species, including *B. cereus* (n=7), *S. pseudintermedius* (n=7), *S. caledonicus* (n=2), *S. warneri* (n=2), *Actinomyces bowdenii* (n=2), *Bacillus pumilus* (n=1), *Bacillus safensis* (n=1), and *Paenibacillus polymyxa* (n=1). These strains were selected based on the location of the canine where the organism was isolated, colony morphology, cross-immunity profile and activity against *L. lactis* HP in original overlay assays. There were multiple bacteriocin operons from Classes I and II within these 22 genomes as identified by BAGEL4 and antiSMASH7.0 (Table 3). Of these 22 genomes, eight were selected for further analysis based on other factors. These include the initial antimicrobial profiles observed when genomes were put through the RiPP and secondary metabolite software tools, the average nucleotide identity (ANI) of genomes of the same species (calculated by EZBioCloud [https://www.ezbiocloud.net/tools/ani]) (data not shown).. The genomes of these eight bacterial isolates were further studied for novel bacteriocin operons by *in silico* analysis with BAGEL4 and antiSMASH7.0, and comparison to closely related bacteriocins based on BLAST results and sequence alignment. Within the eight isolates, a total of 14 putative novel

bacteriocins were identified which are presented in Table 4 with their predicted leader and core amino acid sequences, predicted mass and the online tool utilised, if applicable.

While this study aimed to find putative novel bacteriocins from canine sources it was noted that some of the antimicrobial-producing organisms were of the same pathogenic species associated with animal health from an AMR perspective, e.g. *Staphylococcus pseudintermedius*. Therefore, all genomes sequenced in this study were tested for the presence of antimicrobial resistance and virulence genes using the Comprehensive Antibiotic Resistance Database (CARD) and Virulence Factor Database (VFDB), respectively, with set parameters of 80% identity and coverage through the ABRicate software tool on Galaxy (version 23.1.rc1) (Table 2). Briefly, 12 of the 22 genomes encoded resistance to penam antibiotics due to the presence of the gene BcI/BcII (*B. cereus*), BPU-1 (*B. safensis*), *blaZ* (*S. caledonicus*) and *mgrA* (*S. caledonicus* and *S. warneri*). Resistances to cephalosporins and macrolide antibiotics were the second most abundant class with ten isolates encoding resistance based on the presence of *mefE*, *ermA*, *mphC* and *msrA* genes. The remainder of ARGs reported and the number of isolates with resistance determinants were glycopeptides and fosfomycin (n=7 each), acridine dyes, fluoroquinolones, peptide antibiotics, tetracyclines, and lincosamides (n=3), and fusidic acid and rifamycin (n=1). Interestingly, no ARGs or virulence factors could be found in the two genomes of *A. bowdenii* (APC 4154 & APC 4158), as well as the *B. pumilus* isolate (APC 4140) based on the parameters set in the software tools used. Virulence genes detected using the VFDB software revealed nine virulence genes translating to the following virulence factors: metalloproteases, non-hemolytic enterotoxin A, B and C, hemolysin BL binding component precursor, hemolysin BL lytic component L1 and L2, cytotoxin K and thiol-activated cytolysin. These virulence genes were only detected in the genomes of *Bacillus cereus* isolates. Based on these results, it is unlikely that these antimicrobial-producing strains could be used as

probiotics, other than *A. bowdenii* (APC 4154) and *B. pumilus* (APC 4140). The AMR and virulence genes detected are summarised in Table 2.

Spectrum of inhibition of putative novel bacteriocin-producing isolates

A total of eight antimicrobial-producing isolates from this study were tested against eight gram-positive and seven gram-negative indicators of interest (Table 1). The producers include *S. pseudintermedius, S. warneri, S. caledonicus, A. bowdenii, B. cereus, B. safensis, B. pumilus* and *Paenibacillus sp.* Although *L. lactis* HP was sensitive to all of the bacteriocin producers (consistent with their isolation against this indicator), there was also activity observed against multi-drug resistant (MDR) relevant veterinary pathogens. Of the *Staphylococcus* species identified, *S. pseudintermedius* APC 4170 displayed the greatest spectrum of activity against the indicators tested (5/15), however, the zone of inhibition produced by *S. caledonicus* APC 4137 against indicators *L. lactis* HP and *L. innocua* UCC was larger than that of *S. pseudintermedius* APC 4170 and *S. warneri* APC 4145. *S. warneri* APC 4145 only displayed activity against two of five of the indicators, *M. luteus* APC 4061 and *L. lactis* HP. *A. bowdenii* APC 4154 displayed activity against all eight gram-positive indicators. While the greatest activity observed was against *L. lactis* HP there was also activity against MDR pathogens tested (Table 5). This, in combination with the absence of antibiotic resistance genes and virulence factors (Table 2), within this isolate could be a basis for further testing of the antimicrobial(s) produced and the potential for this strain as a probiotic. Of the *Paenibacillus/Bacillus* isolates, *B. cereus* APC 4133 inhibited the growth of all eight gram-positive indicators tested, while *B. safensis* APC 4157 and *B. pumilus* APC 4140 displayed activity against seven and five of the gram-positive and two and one of the gram-negative indicators, respectively. The *Paenibacillus* isolate also inhibited the growth of all indicators tested. This was not unexpected given the fact

this genus is known to produce multiple antimicrobial compounds including polymyxins and lanthipeptides (Li *et al.*, 2021a). All indicators tested in this assay are listed in Table 1A.

Multiple sequence alignment of novel bacteriocins and operon alignment

Multiple sequence alignment (MSA) of putative novel bacteriocins (Table 4) against known bacteriocins from the same class/subclass was carried out with MUSCLE, and alignments were viewed on Jalview where the percentage identity of bacteriocins was performed via pairwise alignment. The MSAs reported in this study are grouped based on the genus from which the bacteriocins were identified.

In total, seven putative novel bacteriocins were identified within the genus *Bacillus*. These include two circular bacteriocins (*B. pumilus* and *B. safensis*), two lanthipeptides (*Paenibacillus* sp.), and one lasso peptide (*Paenibacillus* sp.). The circular bacteriocin from *B. pumilus* APC 4140, while annotated as butyrivibriocin_AR10 in BAGEL4, was in fact most closely related to gassericin A and acidocin B with 56.9% identity for both when pairwise alignment of the core peptides was calculated. The percentage identity when aligned with butyrivibriocin_AR10 and plantaricyclin A was 51.72% for both peptides and 46.55% when aligned with plantacyclin B21AG. An alignment of these six bacteriocin operons shows the presence of all genes required to translate to proteins responsible for circular bacteriocin synthesis (core peptide, sporulation M protein and ABC transporters) with the exception of one extra transport-related gene within the *B. pumilus* APC 4140 operon, annotated as RND efflux transporter, therefore it could be a potential other transport related protein for the bacteriocin (Figure 3).

Another novel circular bacteriocin was found within the *B. safensis* APC 4157 genome by both BAGEL4 and antiSMASH7.0, with two core peptide genes identified. As this was the only circular bacteriocin identified in this study to contain two putative structural genes, the

structure of the peptides was predicted using ColabFold v1.5.5: AlphaFold2. The product of the first structural gene was found to have a circular shape with 4 alpha-helix domains (consistent with circular bacteriocins), while the second gene product did not yield a circular structure. Due to this and no prior reports of two peptide circular bacteriocin operons, we assigned this gene as having an unknown function (Figure 4). Again, the most closely related bacteriocin was identified via pairwise alignment of core peptides with known circular bacteriocins, and amylocyclicin (40.6%), enterocin-NKR-5-3B (40.6%), and amylocyclicin CMW1 (39.1%) were found to be the most closely related. Whole operon alignments revealed that the bacteriocin operon of APC 4157 matches most closely with enterocin-NKR-5-3B, due to the presence of similar-sized maturation (blue) and ABC transporter related proteins (Figure 4).

Paenibacillus sp. APC 4171 *in silico* analysis identified three putative novel bacteriocins, including two lanthipeptides and one lasso peptide. The lasso peptide was annotated as belonging to the paeninodin family of lasso peptides by BLASTp, and therefore was aligned with this peptide (leader and core). However, following identification of the bacteriocin operon, the presence of genes encoding a nucleotidyltransferase and sulfotransferase were identified in the bacteriocin operon. This was also reported by Zhu and co-workers in *Paenibacillus polymyxa* CR1 (Zhu *et al*., 2016). Due to the 100% amino acid identity between the core peptide of this strain and APC 4171 the bacteriocin operon from strain CR1 and C454 were both used for operon alignment to APC 4171 (Figure 5A). Briefly, pairwise alignment of the prepeptide (leader and core) paeninodin from strain C454 and APC 4150 was calculated as 45.2%, while alignment between APC 4171 and CR1 lasso peptides was 100%. Operon alignment demonstrates all three contain the relative genes required for biosynthesis of the lasso peptide, including maturation enzymes for relevant modifications (Figure 5A).

Two lanthipeptide operons were also identified within *Paenibacillus* sp. APC4171, one closely related to paenilan and the second most closely related to paenicidin B based on antiSMASH. Following the same procedures used for all other bacteriocins in this study, sequence alignments of the prepeptides (core and leader) with their closest relatives were carried out and percentage identities via pairwise alignments were calculated. The paenilan-like lanthipeptide was found to be 94% identical to paenilan from *Paenibacillus polymyxa* E681, with three amino acid differences across the entire prepeptide (one residue difference in the leader and two in the core peptide). Operon alignments with the paenilan bacteriocin operon revealed that APC 4171 harbours all genes required for synthesis of the lanthipeptide paenilan, including *lanB* and *lanC* encoding the maturation enzymes (Figure 5B). The second lanthipeptide identified in APC 4171 was recognised as having 100% similarity to paenicidin B as determined by antiSMASH, but when aligned with both paenicidin A and B the percentage identity of these prepeptide sequences in comparison to the lanthipeptide in APC 4171 was only 44.1% and 47.5% identity to these peptides, respectively. Operon alignment again showed the lanthipeptide from APC 4171 contained all relevant genes for biosynthesis, including *lanB* and *lanC*. The presence of two transposase genes was also noted, one upstream and the other downstream of *lanBC* (Figure 5C).

Three putative novel bacteriocins were identified based on the presence of a prepeptide gene from three different *Staphylococcus* species, two of which were circular bacteriocins and one lasso peptide. While the lasso peptide was annotated as a benenodin-family lasso peptide, its operon did not have the typical makeup of a lasso peptide gene operon, particularly in the lack of a gene encoding the cyclase involved in macrolactam ring formation. Therefore, this bacteriocin was not investigated further following sequence alignment with benenodin-1 (calculated as 20%) (Figure 6A). The circular bacteriocin identified from *S. pseudintermedius* APC 4170 harbours a 70 amino acid core peptide with a three amino acid leader sequence

(Figure 6A/Table 3). Following MSA with core peptides of all known circular bacteriocins the most closely related to this novel peptide was cerecyclin, also a 70 amino acid core peptide but with four amino acids comprising the leader sequence. Pairwise alignment of these two core peptides was calculated as 48.6%. Gene operon comparison demonstrated that the operon from APC 4170 harbours the five genes typically found within circular bacteriocin operons. These include a gene encoding the prepeptide, two genes correlating to an ATP binding protein and transporter permease (ABC transporter) with predicted function reported for secretion/immunity, a second, smaller, hydrophobic putative immunity protein gene, and most importantly the putative maturation enzyme gene (sporulation M protein) for cyclisation of the N to C terminus or the core peptide. Although the cerecyclin operon is reported to contain a transcriptional regulator this was not observed within the operon for APC 4170 (Figure 6B). The third putative novel bacteriocin from *Staphylococcus* is a circular bacteriocin from *S. caledonicus* APC 4137 which was selected for more in-depth characterisation and these results are described in more detail below.

It is worth noting for this study circular bacteriocins were aligned with core peptides only, due to the varying length of leader sequences across all circular bacteriocins (between 2-48 amino acids) (Perez *et al*., 2018).

In silico **analysis, partial purification, and characterisation of caledonicin from *S. caledonicus* APC 4137**

In silico *analysis*

Following *in silico* analysis of the whole genome sequence a putative novel circular bacteriocin from *S. caledonicus* APC 4137 was identified by BAGEL4. The circular bacteriocin core peptide was found to be most closely related to enterocin-NKR-5-3B following pairwise

alignment (39.1% identity) (Figure 7C), and the operons of these two bacteriocins were aligned using the clinker tool on CAGECAT (Gilchrist and Chooi, 2021).

Operon alignment revealed the caledonicin operon to possess all the genes required for circular bacteriocin synthesis as observed in enterocin-NKR-5-3B operon (Figure 7C), with the addition of five genes identified by BAGEL4 upstream from the prepeptide gene. Four of these five genes correspond to an ABC transporter permease, ATP binding protein, putative efflux system protein and one unknown function prediction, which have also been identified in enterocin AS-48 (*as-48EFGH*) (Diaz *et al.*, 2003) and carnocyclin A (*cclEFGH*) (van Belkum, Martin-Visscher and Vederas, 2010). Due to enterocin AS-48 being the representative circular bacteriocin among this subclass, the *as-48EFGH* genes were aligned with these additional genes found using CAGECAT. CAGECAT demonstrated there was similarity between two of the four genes in the caledonicin operon with *as-48G* and *as-48H*. These genes were aligned in BLASTp with the corresponding caledonicin genes. The ATP binding protein, *as-48G* shared 62.7% amino acid identity to the predicted ATP binding protein in the caledonicin operon, while the membrane-spanning protein, *as-48H* and the predicted permease protein shared 37.6% identity (Figure 7C). This shared percentage identity with *as-48G* and *as-48H* leads us to believe these four additional genes upstream of the caledonicin prepeptide correspond to an additional transporter system. Alignment of the enterocin AS-48 biosynthetic operon, *as-48C1DD1*, was also performed but showed no percentage identity to the caledonicin operon, and therefore was omitted from the study. Indeed, future assays would need to be carried out to confirm the effects of the additional proteins on bacteriocin synthesis and immunity, as has been conducted on the enterocin AS-48 and carnocyclin A operons (Diaz *et al.*, 2003; van Belkum, Martin-Visscher and Vederas, 2010). Interestingly, one gene was absent within the caledonicin operon when compared to enterocin NKR-5-3B. This gene is annotated as the enterocin NKR-5-3B immunity protein, *enkB4*. However, a gene was identified by

BAGEL4 upstream of the prepeptide gene and downstream of the predicted transporter cluster discussed above. The amino acid sequence of this gene was put through the ExpasyProtparam online tool and found to be a small (67 amino acids), cationic (net charge +6) and hydrophobic protein (GRAVY index 1.243); all characteristics that correlate to the immunity protein of circular bacteriocins (Perez *et al*., 2018). Therefore, this gene is predicted to be the caledonicin immunity gene (Figure 7C). Based on these results it can be assumed the caledonicin operon displays amino acid identities across two circular bacteriocin operons, enterocin-AS-48 and enterocin-NKR-5-3B (Figure 7C). Caledonicin was subjected to partial purification which was carried out on freeze-thaw samples previously shown to exhibit antimicrobial activity against *L. lactis* HP (data not shown). Partial purification was carried out using a 60mL Strata C18-E column. The bacteriocin was eluted in concentrations of IPA between 50-70% and tested for activity *via* WDA, where the 70% IPA eluent was found to be active against *L. lactis* HP. This 70% IPA eluent was subjected to MALDI TOF mass spectrometry and a strong signal mass of 6077.18 Da was observed (Figure 7D), which correlated perfectly with the predicted mass of 6,077.1 Da. MALDI TOF mass spectrometry also revealed masses potentially correlating to some oxidised caledonicin (+16 Da) and the sodium adduct ion (+22 Da). The doubly-charged caledonicin ion (3,038.13 Da) was also observed (Figure 7D). The predicted structure of the circular bacteriocin was generated through ColabFold v1.5.5: AlphaFold2. This was followed by visualisation of the bacteriocin structure in the PyMOL Molecular Graphics System, Version 3.0. Percentage confidence of this structure is indicated by dark blue regions where pLDDT is >90 (Figure 7E).

Physicochemical assays

Further tests were performed with the semi-purified active fractions, including exposure to proteases for three hours, and heat stability at a range of temperatures (37, 65, 80, 100 and 120

°C for 15 min). Protease treatments revealed the antimicrobial present in the active fraction to be proteinaceous in nature as it was degraded completely by proteinase K at a high concentration 20 mg mL^{-1} compared to untreated controls. The fraction was also subjected to the proteases proteinase K, pepsin, trypsin, and α-chymotrypsin (at lower concentrations of 100 µg mL^{-1}) and shown to be somewhat protease stable since only a partial reduction in antimicrobial activity compared to the untreated control was observed (Figure 8A). Heat treatment of the fraction demonstrated the antimicrobial to be heat stable at all temperatures tested as would be expected for bacteriocins (Figure 8A). This is the first evidence of a circular bacteriocin produced by a *Staphylococcus* species.

Spectrum of inhibition of caledonicin

The antimicrobial activity of semi purified caledonicin was assessed via WDA against all gram-positive indicators listed in Table 1A in addition to a panel of four human gut strains with an added *C. difficile* strain to assess the spectrum of activity of caledonicin against relevant veterinary and human-related pathogens (Table 1B). Antimicrobial activity was observed against all gram-positive indicators listed in Table 1A, excluding *L. bulgaricus* LMG 6901. The highest activity was observed against *L. lactis* HP, as expected given the sensitivity of this strain to other bacteriocins including nisin and lacticin 3147, with inhibition of growth also observed for *M. luteus* APC 4061 and *L. innocua* UCC. Strong inhibitory zones were also noted against a food-associated pathogen, *L. monocytogenes* EGD-e. Inhibition of the veterinary-related *S. pseudintermedius* pathogens (Des Field, Gaudin, *et al.*, 2015), DK 729 and DSM 21284 strains was also observed which was surprising given the poor activity observed by the producing strain in colony-based deferred antagonism assays (Table 5). This increased activity in the WDA could be due to the increased concentration of the bacteriocin from the producing strain. Further WDAs of this fraction were carried out on a panel of human gut isolates (Ríos

Colombo *et al.*, 2023a). Results showed *M. gnavus* ATCC 29149 exhibited the most sensitivity to caledonicin., however, there was slight inhibition of growth of *Lactiplantibacillus plantarum* WCFS1, *Bifidobacterium longum* ATCC 15707 and the human gut pathogen *C. difficile* APC 43 (Figures 7 & 8B).

Discussion

AMR has been described as an ongoing threat to human and animal health, with a prediction of approximately 50 million AMR-related deaths per year by 2050 (O'Neill, 2019). Antimicrobial-resistant bacteria have been in existence long before the discovery of the first clinical antibiotic, penicillin. For example, genes encoding penicillin resistance were found in a *Staphylococcus* strain isolated in 1940, three years before its first use as a human-approved therapeutic in 1943 (Lee Ventola, 2015). The association between antibiotic use and the development of resistance in humans has been highlighted by many health regulatory bodies and emphasises the need for new antimicrobial development if we wish to combat this issue.

Despite the evidence of increased antibiotic resistance, the global consumption of antibiotics by humans and animals is expected to increase by 200% by the year 2030, if no immediate action is taken (Klein *et al.*, 2018; Iriti, Vitalini and Varoni, 2020). Additionally, an increase of 67% in livestock antibiotic consumption is expected to occur between 2010 to 2030 in the US (Van Boeckel *et al.*, 2015). O' Neill highlighted in 2019 that the economic burden relating to the overuse or misuse of antibiotics will mean that 100 trillion US dollars will be lost in global production (O'Neill, 2019). A 2020 review published by the WHO on antimicrobial resistance concluded that the current pipeline of antibiotic development is "dominated" by improving existing classes of antibiotic combinations. However, they also highlighted the importance of development of "newer classes of antibacterials, including antibacterials

addressing new targets and using new modes of action" to overcome AMR (World Health Organisation, 2020).

Another report by the Health Products Regulatory Authority (HPRA) in Ireland stated that in 2020 there were 103.9 tonnes of veterinary antibiotics prescribed in Ireland, though there was a noticeable decrease in 2021 and 2022 to 94.2 and 76.5 tonnes, respectively. This report also noted a steady decrease in the sale of the Category B "Restricted" antibiotics including 3rd and 4th generation cephalosporins, quinolones, and macrolides; and no sale of polymyxins in 2022. (Health Product Regulatory Authority, 2022). It is expected by the HPRA and EU that during the course of 2023 and 2024, further restrictions on veterinary antibiotics will be put in place, in particular in livestock. These include no prophylactic use of antibiotics other than exceptional cases where the risk of infection is very high, and consequences of infection are severe and metaphylactic treatment of animals may only occur after diagnosis of infection of the originally infected animal where risk of spread of the infection is high and no other alternative treatments are available.

Worryingly, there is potential for cross-contamination of AMR bacteria not only between livestock and humans but also within companion animal settings. Transfer of commensal bacteria from pets and humans has been shown to be a two-way system, where it was highlighted that dog ownership resulted in a higher diversity of bacteria on the skin (hands and forehead) of dog owners compared to those without dogs (Song *et al.*, 2013). However, transmission of AMR bacteria between pets and humans can also occur as a result of general contact with the animal such as petting, in particular if living in the same household as the animal; or physical trauma/injury (e.g. dog bite) (Caneschi *et al.*, 2023). Another method of drug-resistant bacterial transfer is via transformation, conjugation, or transduction of bacterial DNA from one strain to another from owner to pet, or *vice-versa* (Rendle and Page, 2018). For

example, Tóth and co-workers detected genes from bacterial species in canine saliva conferring resistance to thirteen different classes of antibiotics and determined the bacteria harbouring these genes, and the genes themselves, are capable of transferring and establishing themselves in humans and their bacteriome (Tóth *et al.*, 2022). This two-way transfer in combination with the ongoing threat and reported health risk of potential AMR and ARG transmission between owners and their pets suggests a need for novel antimicrobials for use in both companion animals and humans.

While stricter regulations regarding the use of current antibiotics may alleviate bacterial resistance development, another approach is to discover new and alternative antibacterial compounds to antibiotics. One such alternative is bacteriocins, ribosomally synthesised antimicrobial peptides (AMPs) produced by bacteria that target closely related strains (Des Field *et al.*, 2015).

Antimicrobial activity of bacteriocins was first observed between strains of *E. coli* producing colicins almost a century ago (Yang *et al.*, 2014) These AMPs are described as a defense mechanism for bacteria to thrive in their environment and reduce competition against related bacteria for nutrients (Yang *et al.*, 2014).

Bacteriocins exhibit potent antimicrobial activity, in particular against AMR pathogens, highlighting their potential as alternatives to antibiotics (Meade, Slattery and Garvey, 2020). While several different environmental niches have been investigated in the past for novel bacteriocins, for example, human and animal gut isolates, skin, and breast milk (Birri *et al.*, 2010; Han *et al.*, 2014; O'Sullivan *et al.*, 2019; Angelopoulou *et al.*, 2020; Sugrue *et al.*, 2020; Wosinska *et al.*, 2022; Uniacke-Lowe *et al.*, 2023), to our knowledge, none have involved canine sources with the exception of one study which focused specifically on enterococci from canine faeces and their antimicrobial potential against veterinary related pathogens. This study however did not aim to screen for novel bacteriocins (Kubašová *et al.*, 2020). With that in

mind, a screening study was designed and implemented to identify putative novel bacteriocins from canine sources.

Over 5,000 bacterial isolates from five canines were tested for antibacterial activity against a standard sensitive indicator, *L. lactis* HP. From these initial results, colonies of different morphologies and producing phenotypically different inhibitory spectra against the indicator strain were selected for further testing. The majority of antimicrobial-producing bacteria of all sites tested in this study were isolated from the gum with crossover of bacterial species found between this area and their skin and/or nose based on 16S rRNA sequencing and WGS results; understandably so as dogs can transfer bacteria from different areas of their bodies due to their grooming behaviour. A total of 42 isolates across the five canines were selected for further testing of cross-immunity and 16S rRNA sequencing. Based on the results obtained, 22 bacterial isolates of different genera and species were identified and selected for WGS. More specifically, of the 22 WGS isolates, the most abundant bacteriocin producers were isolated from the mouth (14/22, 63.6%), followed by the nose (4/22, 18.2%), and lastly the axilla and ear (2/22 each, 9.1%). Notably, the ears proved to be a poor source of bacteriocin-producing strains, where the only antimicrobial producers from this site were found in two of five dogs (*Paenibacillus* sp. APC 4171 and *B. safensis* APC 4157). This low abundance from this site could be due to the erect shape of the dog's ears which participated in this study; a trait which is thought to prevent overgrowth of bacteria or yeast due to low moisture and increased air circulation (O'Neill *et al.*, 2021).

These 22 genomes were screened for their bacteriocinogenic potential and also the presence of antimicrobial resistance genes (ARGs) and virulence factors to determine their suitability for probiotic use. Results of ARG and virulence factor presence in the WGS isolates in this study show there are ARGs present in almost all isolates, except for *A. bowdenii* APC 4154 and *B.*

pumilus APC 4140, based on parameters set in the CARD and VFDB databases. This suggests these two strains could potentially have probiotic potential for companion animals (Table 2).

Upon further *in silico* and *in vitro* investigation the number of isolates selected for in-depth analysis was reduced to eight following further assessment for duplications of strain/species. The European Food and Safety Authority Panel on Animal Health and Welfare (AHAW) reported in 2021 a target working group of bacterial pathogens for AMR in dogs and cats, including *Enterococcus faecalis, E. coli, K. pneumoniae, P. aeruginosa, S. pseudintermedius* and *S. aureus* (Nielsen *et al.*, 2021). Based on these reported target pathogens, antimicrobial-producing isolates from this study were tested for antibacterial activity against a range of strains from the species mentioned above and others, including *L. monocytogenes* and *Salmonella enterica* serovar Typhimurium. Based on inhibition results where each antimicrobial producer tested exhibited activity against two or more indicators tested, further characterisation of these isolates and their antimicrobials was carried out, including purification and characterisation, and specific activity against a greater range of AMR pathogens.

A total of 14 putative novel bacteriocins were identified from eight WGS bacterial isolates via *in silico* mining for prepeptide bacteriocin genes using BAGEL4, antiSMASH7.0 or Artemis (Table 4), from all five canines. Of these 14 bacteriocins, one particular bacteriocin of interest was a circular bacteriocin from strain *S. caledonicus* APC 4137, a previously identified novel species also isolated from a canine source (Newstead *et al.*, 2021); however, no bacteriocins have been identified from this species to date. Based on this novel circular bacteriocin (termed caledonicin) being the first identified from this bacterial species, further tests were carried out to characterise the antimicrobial further. Circular bacteriocins are class IIc bacteriocins, so named due to their N to C terminal covalent linkage resulting in the formation of a ring structure. While these bacteriocins are currently classified as unmodified, it has been suggested

they be reclassified to the modified, class I bacteriocin grouping due to their simple, yet modified structure (Alvarez-Sieiro *et al.*, 2016; Pérez-Ramos *et al.*, 2021; Sugrue, Ross and Hill, 2024). To date, around 20 circular bacteriocins have been identified with enterocin AS-48 as the representative bacteriocin for this class (Galvez *et al.*, 1986). While these peptides share very little amino acid sequence similarity amongst each other, with leader sequences ranging between 2-48 amino acids, they do share characteristics such as thermal stability and protease resistance. These peptides have been distinguished further into two subgroups (I and II) by Perez and co-workers where subgroup I are highly cationic and have a general isoelectric point (pI) greater than 9, while subgroup II are highly hydrophobic, and their pI is <7 (Perez *et al*., 2018). Operons of circular bacteriocins generally contain genes encoding (i) the prepeptide, (ii) a maturation enzyme (sporulation M protein), (iii) an ABC transporter consisting of an ATP binding domain and transmembrane permease for transportation/immunity purposes, and (iv) a small, hydrophobic immunity protein (Perez *et al*., 2018). Circular bacteriocins are gaining more attention in the bacteriocin field and can be identified via *in silico* analysis of bacterial genomes in high abundance, as shown by Xin and co where nearly 7,000 putative circular prepeptide genes were identified across 86 species (Xin *et al.*, 2020). This study has expanded further the class IIc bacteriocins discovered to date, with the identification of four novel circular bacteriocins in this study via *in silico* analysis, and semi-purification and characterisation of one of these four peptides. Given the success of identifying novel bacteriocin producers from aerobic environments from canines in this study, further studies should be conducted on other areas such as the gut microbiome of these animals and other pets to tackle AMR.

We describe caledonicin, a novel circular bacteriocin from *S. caledonicus* APC 4137 comprising a 64 amino acid core peptide based on structural characterisation (Figure 7E). Partial purification of the antimicrobial and subsequent MALDI TOF mass spectrometry confirmed the presence of the bacteriocin (6,077.18Da) in the purified fraction (Fig. 7D).

Pairwise alignment and bacteriocin operon alignment to enterocin-NKR-5-3B was also performed. Caledonicin is a subgroup I, heat-stable, protease-stable, circular bacteriocin that most closely resembles enterocin-NKR-5-3B based on percentage identity of the core peptide. Interestingly the bacterial strain when tested against indicators of interest in overlay assays only exhibited activity against *L. lactis* HP, *L. innocua* and *M. luteus* (Table 4). However, following WDA with semi-purified bacteriocin, caledonicin was found to inhibit the growth of a range of gram-positive pathogens, including the MRSP pathogens *S. pseudintermedius* DK 729 and DSM 21284, and the food-related pathogen *L. monocytogenes* EGD-e. Of the five SIHUMI strains tested, *R. gnavus* ATCC 29149 exhibited the most sensitivity to caledonicin., followed by slight inhibition of growth of *Lb. plantarum* WCFS1, *B. longum* ATCC 15707 and *C. difficile* APC 43. While *R. gnavus* ATCC 29149 is considered a human gut commensal it has been associated with gut-related disorders/diseases, including irritable bowel disease/syndrome (IBD/IBS) and Crohn's disease (CD) when present in high abundance (Crost *et al.*, 2023) (Figure 8B). This lack of activity from overlay assays in comparison to semi-purified bacteriocin could be due to low concentrations in a bacterial culture and greater activity when concentrated.

In summary, these results demonstrate the promising properties of caledonicin in terms of stability and antimicrobial activity and suggest that this peptide merits further investigation as a novel antimicrobial alternative for both human and veterinary applications.

Conclusion

Resistance to antibiotics is a serious threat and their indiscriminate use has led to management restrictions in humans and animals. Bacteriocins are peptides synthesized by bacteria that can kill or inhibit the growth of other bacteria which makes them invaluable for food preservation and potential therapeutic applications. In this study, we carried out the first bacteriocin screening study involving bacterial strains from canines and identified 14 putative novel bacteriocins based on prepeptide analysis of 22 bacterial isolates using whole genome sequencing. One of these bacteriocins, caledonicin, is the first novel bacteriocin identified and characterized from *S. caledonicus*. It appears that the microbiome of canines represents an as-yet untapped but rich source of bacteriocin-producing bacteria and based on the number of hits for antimicrobial compounds identified in this study on a sample size of only five canines, these animals are a worthy niche that warrants further investigation for antibiotic alternatives in the fight against AMR pathogens.

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Tables and Figures

Table 1. Bacterial indicator strains and their growth conditions used in this study.

A: Species/strain	Conditions
Lactococcus lactis subsp. *cremoris* HP	GM17, aerobic, 30 ˚C
Micrococcus luteus APC 4061	BHI, aerobic, 37 ˚C
Staphylococcus pseudintermedius DK 729	BHI, aerobic, 37 ˚C
Staphylococcus pseudintermedius DSM 21284	BHI, aerobic, 37 ˚C
Listeria innocua UCC	BHI, aerobic, 37 ˚C
Listeria monocytogenes EGDe	BHI, aerobic, 37 ˚C
MRSA DPC 5645	BHI, aerobic, 37 ˚C

Enterococcus faecalis VRE V583	BHI, aerobic, 37 ˚C
Cronobacter sakazakii DPC 6440	LB, aerobic/anaerobic, 37 ˚C
Salmonella enterica serovar typhymurium UK1	LB, anaerobic/aerobic, 37 ˚C
Klebsiella pneumoniae NCIMB 13218	LB, anaerobic/aerobic, 37 ˚C
Escherichia coli ETEC K88f4	LB, anaerobic/aerobic, 37 ˚C
Escherichia coli ETEC F18ab	LB, anaerobic/aerobic, 37 ˚C
Escherichia coli K12 MG1655 (ATCC 47076)	LB, Anaerobic/aerobic, 37 ˚C
Pseudomonas aeruginosa PA01	BHI, aerobic, 30 ˚C

Strains listed below were only tested against the circular bacteriocin, caledonicin identified in this study.

B: Species/Strain	Conditions
Lactobacillus delbrueckii subsp. *bulgaricus*	MRS, anaerobic, 37 ˚C
Enterococcus faecalis OG1RF	LYHBHI, anaerobic, 37 ˚C
Lactiplantibacillus plantarum WCFS1 (NCIMB 8826)	LYHBHI, anaerobic, 37 ˚C
Bifidobacterium longum ATCC 15707	LYHBHI, anaerobic, 37 ˚C
Mediterraneibacter gnavus ATCC 29149	LYHBHI, anaerobic, 37 ˚C
Clostridioides difficile APC 43 (ATCC 43255)	LYHBHI, anaerobic, 37 ˚C

ATCC, American Type Culture Collection; APC, APC Microbiome Ireland Culture Collection; DPC, Teagasc Culture Collection; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; DSM, Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures; UCC, University College Cork, Cork, Ireland; NCIMB, National Collection of Industrial, Food and Marine Bacteria. LB; Luria-Bertani, BHI; Brain Heart Infusion, LYHBHI; BHI medium supplemented with 0.5% yeast extract (Difco), 5 mg/liter hemin, cellobiose (1

mg/ml; Sigma–Aldrich), maltose (1 mg/ml; Sigma), and cysteine (0.5 mg/ml; Sigma), GM17; M17 supplemented with 0.5% glucose.

Table 2. Antibiotic resistance genes (ARGs) and virulence factors identified in twenty-two genomes analysed in this study.

Strain ID	Species	AMR/Virulence genes (80% identity) as identified by CARD/VFDB	Antibiotic class conferred resistance to/Virulence factors
APC 4130, 4133, 4148, 4149, 4160, 4164, 4166	*Bacillus cereus*	*inhA*	Immune inhibitor A metalloprotease
		nheA	Non-hemolytic enterotoxin A
		nheB	Non-hemolytic enterotoxin B
		nheC	Non-hemolytic enterotoxin C
		hblA	Hemolysin BL binding component precursor
		hblD	Hemolysin BL lytic component L1
		hblC	Hemolysin BL lytic component L2
		cytK	Cytotoxin K
		BAS3109	Thiol-activated cytolysin
		Bcl/BcII	Cephalosporin/Penam

Table 2. continued

APC 4130, 4133, 4148, 4149, 4160, 4164, 4166	*Bacillus cereus*	*FosB*	Fosfomycin
		vanZF	Glycopeptide
No virulence factors detected for remainder of isolates listed below			
APC 4157	*Bacillus safensis*	*BPU-1 (beta-lactamase)*	Penam
		cat86	Phenicol
APC 4171	*Paenibacillus polymyxa*	*rphB*	Rifamycin
APC 4140	*Bacillus pumilus*	-	-
APC 4136, 4152, 4153, 4156, 4161, 4163, 4170	*Staphylococcus pseudintermedius*	*mefE*	Macrolide
APC 4137, 4147	*Staphylococcus caledonicus*	*PC1_beta-lactamase_(blaZ)*	Penam
		mgrA/norR	Acridine dye Cephalosporin Fluoroquinolone Penam Peptide Tetracycline
		ErmA	Lincosamide Macrolide Streptogramin

Table 2. continued

		mgrA/norR	Acridine dye Cephalosporin Fluoroquinolone Penam Peptide Tetracycline
		norA	Acridine_dye Fluoroquinolone
APC 4145	*Staphylococcus warneri*	*fusB*	Fusidic acid
		qacA	Fluoroquinolone
		mphC	Macrolide
		msrA	Lincosamide Macrolide Oxazolidinone Phenicol Pleuromutilin Streptogramin Tetracycline
APC 4154, 4158	*Actinomyces bowdenii*	-	-

Table 3. Bacteriocin prediction and their classes as annotated by BAGEL4 and antiSMASH7.0 for 22 whole-genome sequenced strains in this study.

Strain ID	Genus/Species	Area (canine isolated from)	Bacteriocin type (class)	
			BAGEL4	antiSMASH7.0
APC 4130	*Bacillus cereus*	Mouth (C1)	Thiopeptide x2 (Class I), Sactipeptide (Class Ic), LAP (Class I)	TOMM (Class I), Thiopeptide x4 (Class I), LAP (Class I), Sactipeptide (Class Ic)
APC 4133	*Bacillus cereus*	Mouth (C1)	Thiopeptide x2 (Class I), Sactipeptide (Class Ic), LAP (Class I)	RRE-containing (no class), TOMM (Class I), LAP (Class I), Thiopeptide x3 (Class I), Sactipeptide (Class Ic)
APC 4136	*Staphylococcus pseudintermedius*	Mouth (C1)	LAP (Class I), non-pediocin like (Class IId), Sactipeptide x2 (Class Ic)	LAP (Class I), Circular bacteriocin (Class IIc), non-pediocin like (Class IId), TOMM (Class I)
APC 4137	*Staphylococcus caledonicus*	Mouth (C1)	Sactipeptide (Class Ic), Circular bacteriocin (Class IIc)	-

Table 3. continued

APC 4140	*Bacillus pumilus*	Mouth (C1)	Circular bacteriocin (Class IIc), Sactipeptide x2 (Class Ic)	RRE-containing (no class), Sactipeptide (Class Ic)
APC 4145	*Staphylococcus warneri*	Mouth (C1)	Non-pediocin like (Class IId), Sactipeptide (Class Ic),	Non-pediocin like (Class IId)
APC 4147	*Staphylococcus caledonicus*	Mouth (C1)	Sactipeptide (Class Ic), Circular bacteriocin (Class IIc)	Circular bacteriocin (Class IIc)
APC 4148	*Bacillus cereus*	Mouth (C1)	Thiopeptide x2 (Class I), Sactipeptide (Class Ic)	Thiopeptide x3 (Class I), Sactipeptide (Class Ic), LAP (Class I), TOMM (Class I)
APC 4149	*Bacillus cereus*	Axilla (C1)	Thiopeptide (Class I), Sactipeptide x2 (Class Ic), LAP (Class I)	Thiopeptide x3 (Class I), TOMM (Class I), Sactipeptide (Class Ic), LAP (Class I)
APC 4152	*Staphylococcus pseudintermedius*	Mouth (C2)	LAP (Class I), circular bacteriocin (class IIc), Sactipeptide x2 (Class Ic)	LAP (Class I), circular bacteriocin x2 (Class IIc), TOMM (Class I)
APC 4153	*Staphylococcus pseudintermedius*	Mouth (C2)	LAP (Class I), circular bacteriocin (Class IIc), Sactipeptide x) (Class Ic)	LAP (Class I), Circular bacteriocin x2 (Class IIc), TOMM (Class I)

Table 3. continued

APC 4154	*Actinomyces bowdenii*	Mouth (C2)	Thiopeptide (Class I), Sactipeptide (Class Ic)	Thiopeptide x2 (Class I)
APC 4156	*Staphylococcus pseudintermedius*	Nose (C2)	LAP (Class I), Circular bacteriocin (Class IIc), Sactipeptide x2 (Class Ic)	LAP (Class I), Circular bacteriocin x2 (Class IIc), TOMM (Class I)
APC 4157	*Bacillus safensis*	Ear (C2)	UviB (x2), LAP (Class I), Circular bacteriocin (Class IIc), Sactipeptide (Class Ic)	LAP (Class I), Circular bacteriocin (Class IIc)
APC 4158	*Actinomyces bowdenii*	Mouth (C3)	Thiopeptide (Class I), Sactipeptide (Class Ic)	Thiopeptide (Class I)
APC 4160	*Bacillus cereus*	Nose (C3)	LAP (Class I), Sactipeptide x2 (Class Ic), Thiopeptides x2 (Class I),	LAP (Class I), Thiopeptide x3 (Class I), TOMM (Class I), Sactipeptide (Class Ic)
APC 4161	*Staphylococcus pseudintermedius*	Nose (C3)	LAP (Class I), Circular bacteriocin (Class IIc), Sactipeptide (Class Ic)	LAP (Class I), Circular bacteriocin x2 (Class IIc), TOMM (Class I)
APC 4163	*Staphylococcus pseudintermedius*	Nose (C3)	LAP (Class I), Circular bacteriocin (Class IIc), Sactipeptide (Class Ic)	LAP (Class I), Circular bacteriocin x2 (Class IIc), TOMM (Class I)

Table 3. continued

APC 4164	*Bacillus cereus*	Axilla (3)	LAP (Class I), Sactipeptide x2 (Class Ic), Thiopeptides x2 (Class I)	LAP (Class I), Sactipeptide (Class Ic) Thiopeptide x3 (Class I), TOMM (Class I)
APC 4166	*Bacillus cereus*	Mouth (C4)	LAP (Class I), Sactipeptide x2 (Class Ic), Thiopeptides (x2)	LAP (Class I), Sactipeptide (Class Ic) Thiopeptide x3 (Class I), TOMM (Class I)
APC 4170	*Staphylococcus pseudintermedius*	Mouth (C5)	LAP (Class I), Circular bacteriocin (class iIc), Sactipeptide x2 (Class Ic)	LAP (Class I), Circular bacteriocin x2 (Class IIc), thiopeptide (Class I)
APC 4171	*Paenibacillus sp.*	Ear (C5)	Lasso peptide (Class I), Lanthipeptide x2 (Class Ia), Sactipeptide x4 (Class Ic)	Lasso peptide (Class I), Lanthipeptide x2 (Class Ia)

Table 4. Putative novel bacteriocins predicted by *in silico* genome mining of WGS bacterial isolates from this study. *Unable to predict leader and core sequence. ND- mass not determined due to intensive modifications and/or unable to distinguish leader from core peptide.

Strain ID	Genus/Species	Area	Bacteriocin	AA sequence (**Leader** & core)	Predicted molecular weight (Da)	Identified by
APC 4154	*Actinomyces bowdenii*	Mouth (C2)	1. Arthropod defensin (x2) 2. Invert defensin containing domain 3. Invert defensin containing domain	1. **MDKFTRRTADLASNDANKALNSET HTPLENAE**GFGCPFSAYECDRHCTSK GYRGGYCRGFVRQTCACY (x2) 2. **MPRFVRRSTALADATFKQALHSET HAPTEGA**EYNCPTDEAPCDRHCRYS GYRGGYCGGMLKASCYCY (x1) 3. **MKKEMHMEIFSRRSRSLSDSRFNDT INSETRSPLETSE**HLSCPFNEHQCYKY CLSKGYRGGYCGGLAFAICRCY (x1)	1. 4044.50 2. 4087.50 3. 4106.60	Artemis
			*Thiopeptide	MNNVIDFAAIEISDLIEDAVDGGELPS QVMAASTTTSGCACSSCSSTCS	ND	BAGEL4 & antiSMASH
APC 4170	*Staphylococcus pseudintermedius*	Mouth (C5)	Circular bacteriocin	**MLN**LHKKIAWTGVKGSAVTSFVSAL ATGSDIWAALTVAGIAFGGGVGTAIA AIGRATVVKFIKRWGVKKAAAW	7051.36	antiSMASH

Table 4. continued

APC 4145	*Staphylococcus warneri*	Mouth (C1)	Lasso peptide	**MTESDFKKYNQLVGDRQLPGDVAQ RT**GHNGVLSDDLMTNKIHYRKSEFSF CL	2994.41	Artemis
APC 4137	*Staphylococcus caledonicus*	Mouth (C1)	Circular bacteriocin	**MSKMKSMSFWTILTIAFFAATAIAL SLANAPF**IAANLGVSSGTAYSMANAL NNISNVATALTIIGTFTGVGTIGSGIAA TILAILKKKGVAAAAAF	6077.1	BAGEL4
APC 4140	*Bacillus pumilus*	Mouth (C1)	Circular bacteriocin	**MRASLILDHLNLSKFESIILAGFFAT AALIGITLN**IGLIADFFGIKIAADWYR QLTDWLAAGGSLTTFAAIVMGVTLPA WLAAAATALGAYAA	5940.96	BAGEL4
APC 4157	*Bacillus safensis*	Ear (C2)	Circular bacteriocin	1: **MMK**VKKLGLISLLLFASTMASLVVS NGSSVATVITSVGVFFGVGIISSGVAA AILAVLKKQGKAKAAAF 2: **MTKATDSKFYALLSLSLLAVTLVAL VIGNGSL**IAANLGVSTATAATVVNFL DTWSSVATVITIVGVFTGVGTISSGVA ATILAILKKQGKAKAAAF	6238.30	BAGEL4 & antiSMASH

Table 4. continued

APC 4133	*Bacillus cereus*	Mouth (C1)	*Heterocycloanthracin family bacteriocin	MEGFIMNQFQQELQSLNLNDYQTG NVVYWDQQQSQYPYYYIQDDARR CGGCGGCGGRCGGCGGRCGGCAG RCGGCIGCAGCFSCFNCWNWWII	ND	antiSMASH
			*Thiocillin family RiPP	1: MNKDLVKSVDNNTQALYIEEQVD ATEFAGFTTAGSVATTSTLSSAGSC GGSFATGSSFSSAG 2: MEELNENIYIEEQDDQLNEVAGTW GSASCFGSFSTFGGCAASASSTATA SSAG 3: MSKLSDSKPTDSAIYLEEQVELNEV AASLGSVSTFSSGSCPGSTVNTVST ASCQG	ND	antiSMASH
APC 4171	*Peanibacillus polymyxa*	Ear (C5)	Lasso peptide	**MSKKEWQEPTIEVLDINQTM**AG KGWKQIDWVSDHDADLHNPS	2536.65	BAGEL4 & antiSMASH
			Lanthipeptide	**MSNNQFDLDVQVSKNVGKIEPQ**V TSVFACTPGCITGPNCGSSECGTVP CGKTTSRLC	3317.95	BAGEL4 & antiSMASH
			Lanthipeptide	**MKNQFDLDLQVAKDEVASKGVQ PA**SGIICTPSCATGTLNCQVSLTFCK TC	2516.00	BAGEL4 & antiSMASH

Table 5. Spectrum of inhibition of putative novel bacteriocin producing isolates from canine sources against indicators of interest via spot assays.

Strain↓/Indicator→	HP	APC 4061	DK729	DSM 21284	DPC 5645	V583	EGDe	UCC	UK1	NCIMB 13218	K88f4	F18ab	MG1655	PA01	DPC 6440
APC 4137 (*S. caledonicus*)	14.4±3.3	4.1±0.2	0	0	0	0	0	11.8±0.6	0	0	0	0	0	0	0
APC 4140 (*B. pumilus*)	18.8±1.1	11.5±1.1	14.2±5.9	11.7±4.2	16.1±4.1	0	0	0	0	0	4.1±0.3	ND	0	0	0
APC 4145 (*S. warneri*)	5.4±0.1	2.9±0.3	0	0	0	0	0	0	0	0	0	0	0	0	0
APC 4157 (*B. safensis*)	20±1.1	15±0.8	20.8±2.3	18±0.4	19.5±1.7	ND	4.8±1.1	14.6±0.4	0	0	4.8±0.2	6.4±4.1	0	0	0
APC 4170 (*S. pseduintermedius*)	6.9±0.8	5.9±0.2	0	0	0	3.6±0.7	5.5±0.8	5.9±0.6	0	0	0	0	0	0	0
APC 4171 (*Paenibacillus sp.*)	18.8±1.3	17.7±1.3	11.7±0.8	13.8±0	12.7±0.3	2.4±0	10.7±0.6	10.3±0.7	15.9±0.8	21.7±0.7	17.7±0.1	10.9±0.4	20.3±0.3	10.7±0.6	16.9±0.1
APC 4133 (*B. cereus*)	21.7±1.1	19.3±0.2	10.4±1.1	8.7±1.3	15±0.4	12.9±0.6	16±1.9	17.1±0.4	0	0	0	0	0	0	0
APC 4154 (*A.bowdenii*)	20.4±1.6	6.7±0.8	5.4±0.4	3.7±0.4	2.5±0.1	5.2±0.5	4.3±0.8	4±0.2	0	0	0	0	0	0	0

Genus and species of indicators are available in Table 1. ND; not determined.

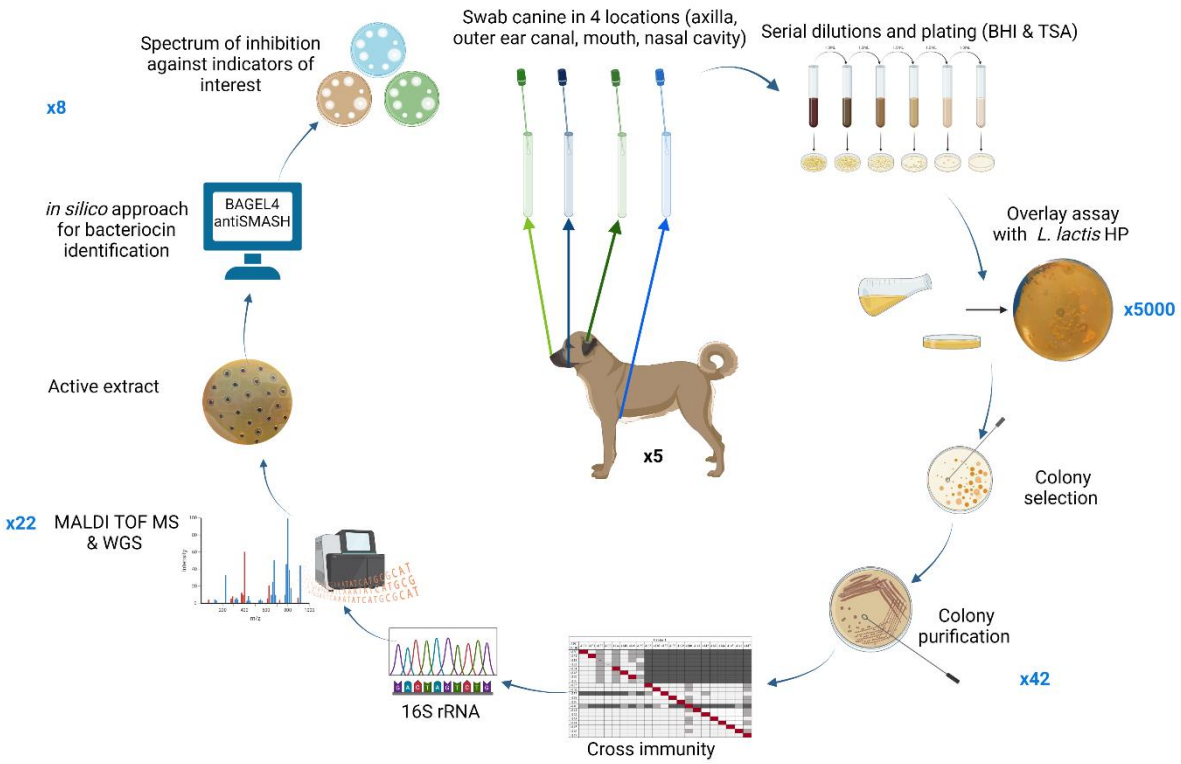


Figure 1. Schematic representation of methods performed in this screening study. Numbers in blue outside the circle represent the number of bacterial isolates brought forward at stages of the screen. Figure created using BioRender.

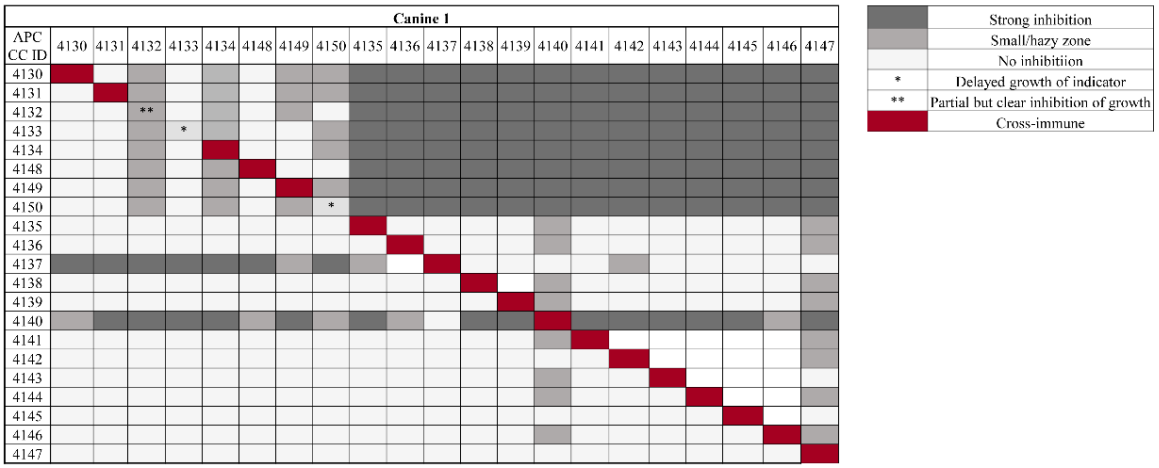


Figure 2. Cross-immunity of antimicrobial producing isolates from one of five canines in this study using overlay assays. Strains listed in the top row are indicators overlaid against antimicrobial producing strains listed on the left column of the table. Strength of inhibition by the antimicrobials produced is indicated in the key (right) where the darker the colour grey, the stronger the inhibition observed. Red diagonal boxes indicate the producer is immune to its own antimicrobial, with the exception of three bacterial isolates for this particular canine, marked with asterisk(s) (*/**). One asterisk (*) represents producers whose antimicrobial delays growth when tested against itself, while two asterisks are representative of the producer exhibiting definite inhibition of growth when tested against itself (**). Cross-immunity of all other canines in this study is available in supplementary material (Figure S1).

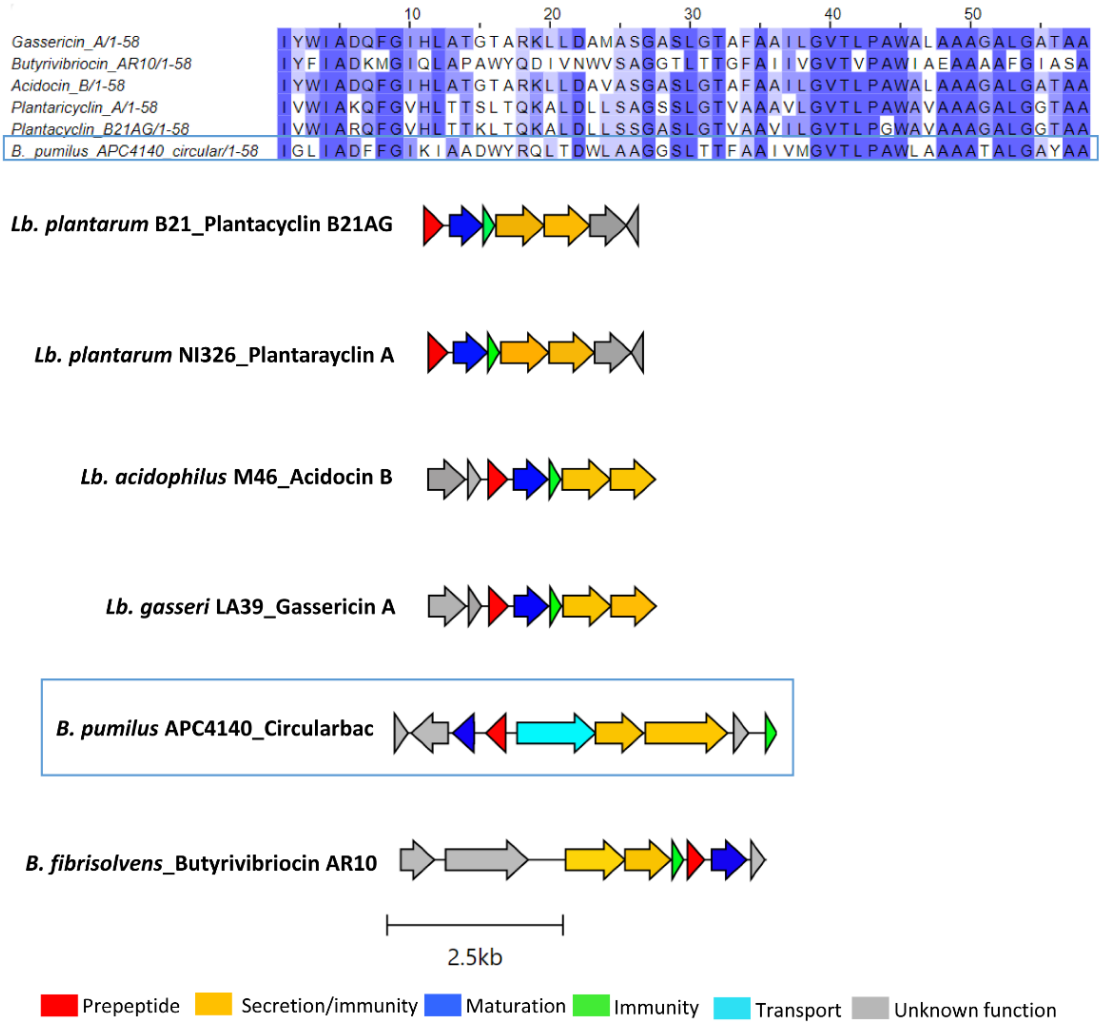


Figure 3. Multiple sequence alignments (MSA) of circular bacteriocin core peptide identified within *B. pumilus* APC 4140 with subgroup II circular bacteriocins, and gene operon alignment for the same. Genbank accession for class IIc bacteriocins in these alignments: Butyriovybriocin_AR10; AF076529.1, Gassericin A; AB007043.2, Acidocin B; KP728900.1, plantacyclin B21AG; CP025732.1, plantaricyclin A; NDXC01000075.1.

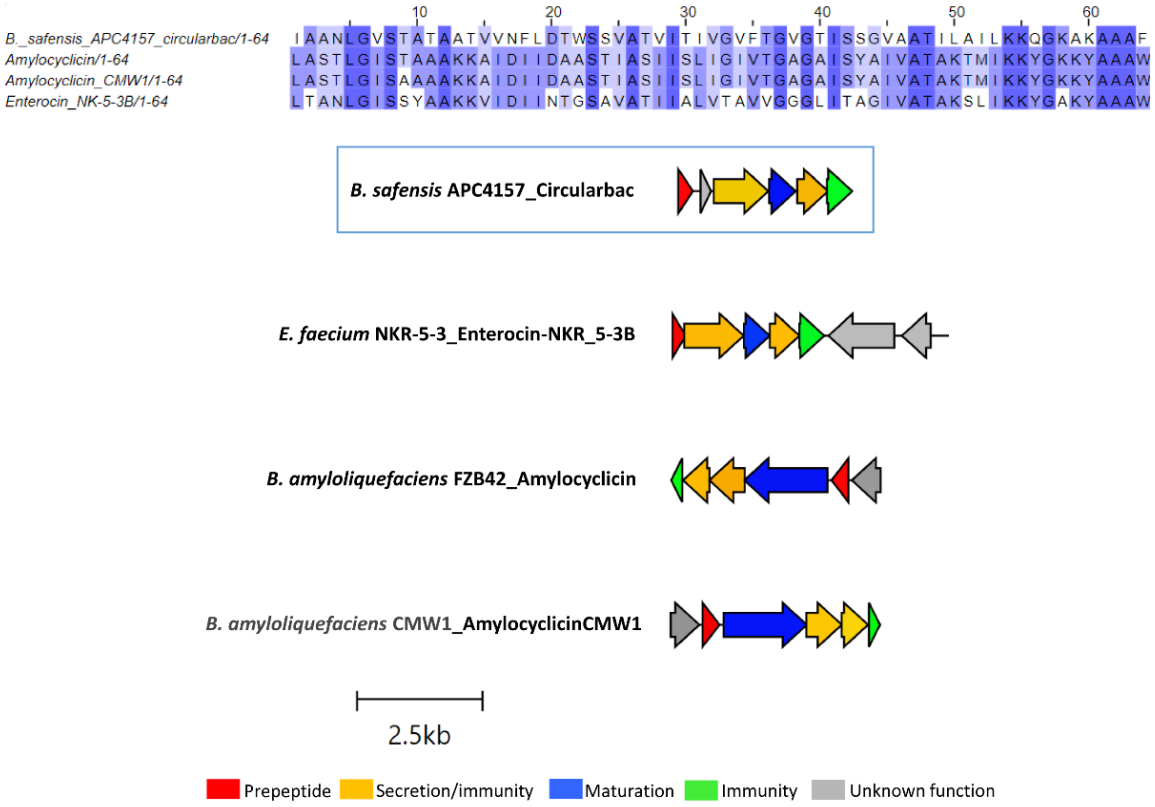


Figure 4. MSA of circular bacteriocin core peptide identified within *B. safensis* APC 4157 with subgroup II circular bacteriocins, and gene operon alignment for the same. Genbank accession for class IIc bacteriocins in these alignments: Amylocyclicin; GCA_000015785.2, Amylocyclicin CMW1; GCA_000747705.1, Enterocin-NKR-5-3B; LC068607.1.

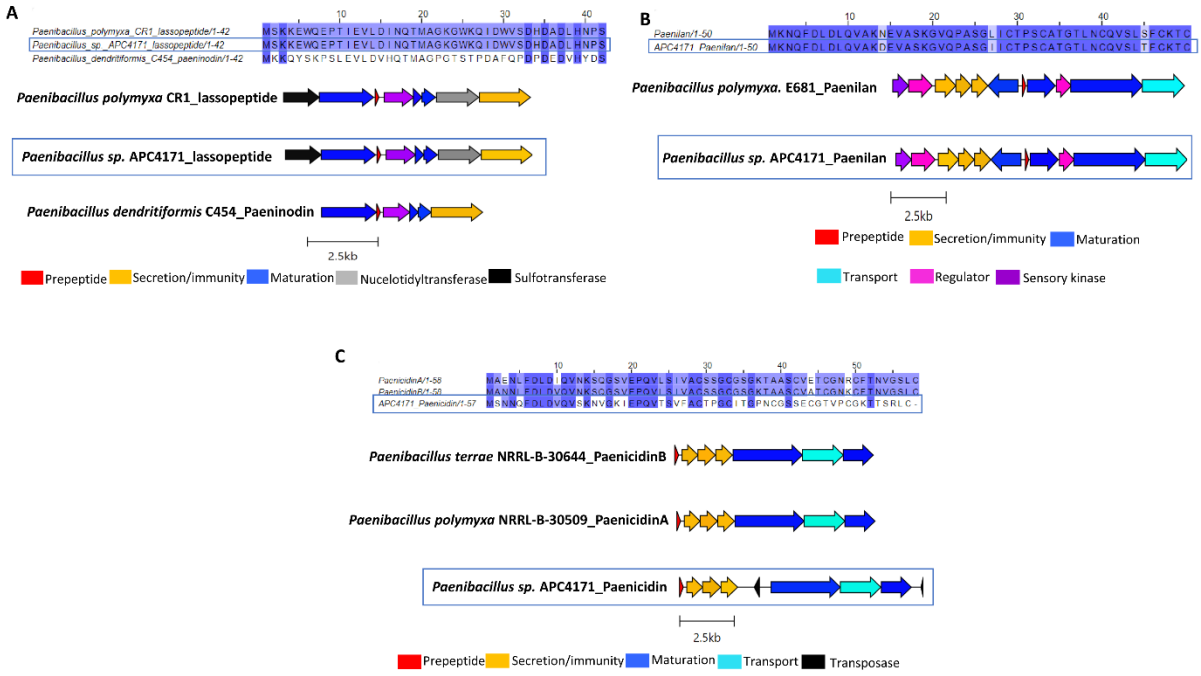


Figure 5. (**A**) MSA of lasso peptide identified within APC 4171 with paeninodin (Genbank accession; GCA_000245555.2) and another lasso peptide identified within another Paenibacillus species (CR1) (Genbank accession; GCA_000507205.2), and gene operon alignment of same. (**B**) MSA of second lanthipeptide identified within APC 4171 with lanthipeptide paenilan (Genbank accession; CP000154) and gene operon alignment of same. (**C**) MSA of lanthipeptide identified within *Paenibacillus* sp. APC4171, with paenicidin A (Genbank accession; JTHO01000011.1) & paenicidin B (Genbank accession; JTHP01000001.1) and gene operon alignment for the same.

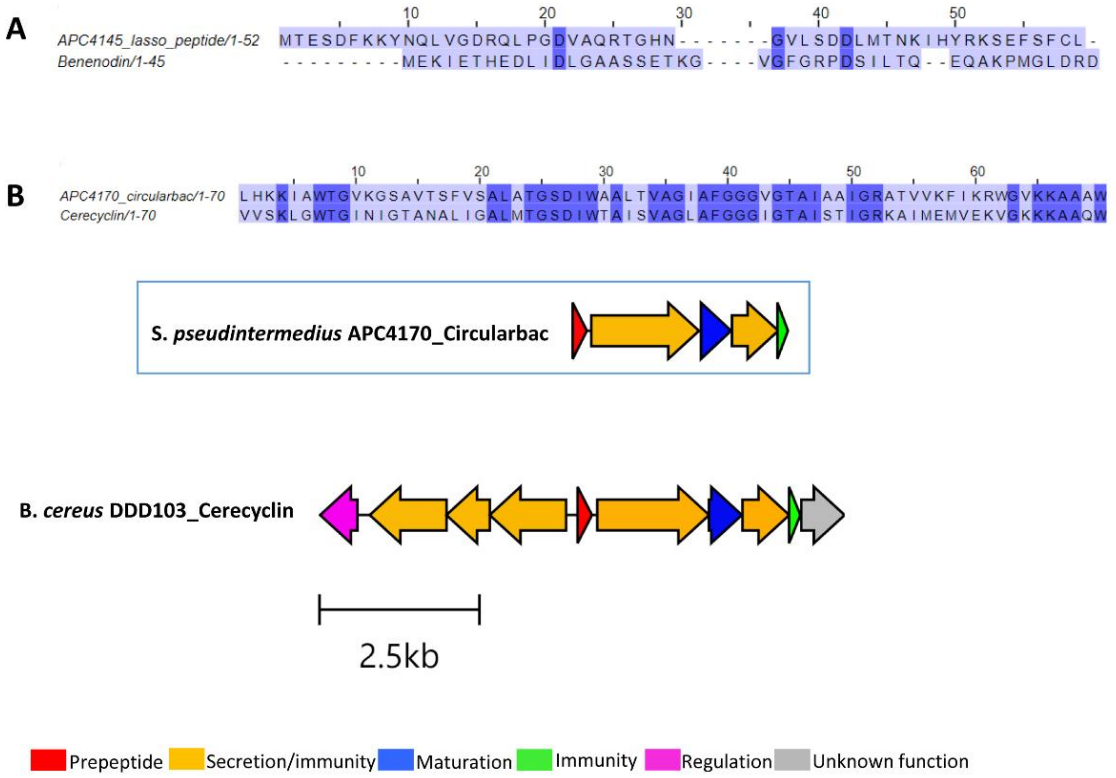


Figure 6. Sequence alignments of bacteriocin peptides identified within (**A**) *S. warneri* APC 4145 and (**B**) *S. pseudintermedius* APC 4170, and the biosynthetic operonfor this circular bacteriocin aligned with most closely related, cerecyclin (Genbank accession; MH037333.1). Only core peptide is aligned for the circular bacteriocin identified in APC 4170. Gene operon alignment was carried out for this same bacteriocin, but not for APC 4145.

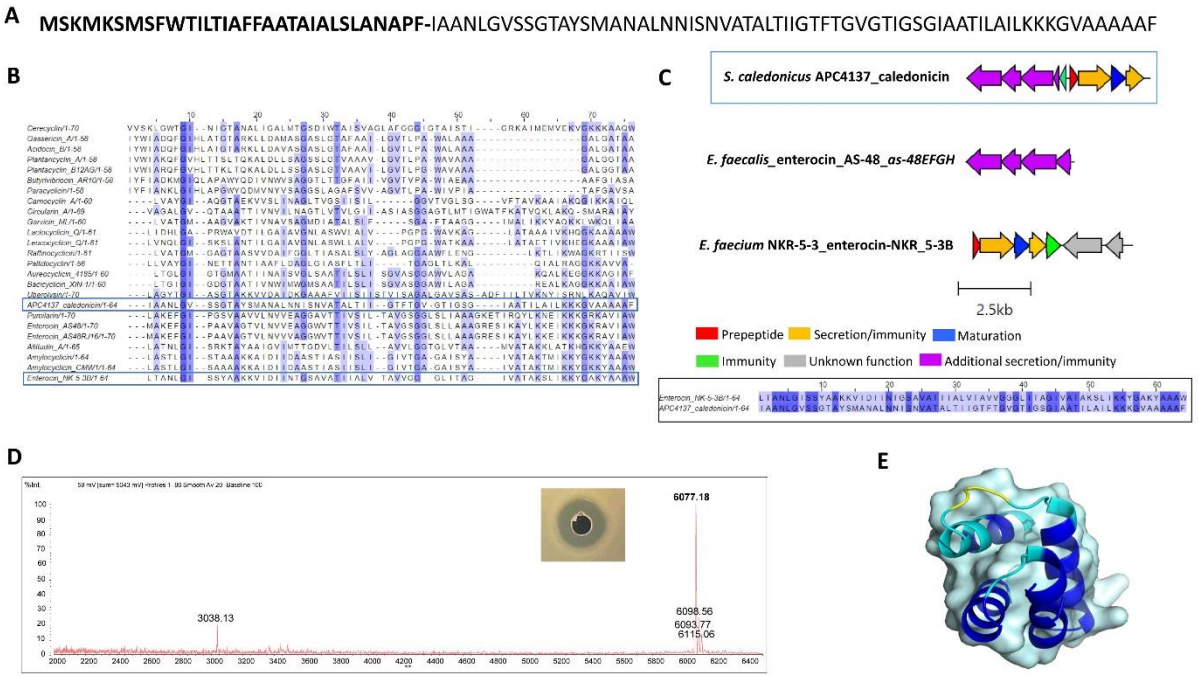


Figure 7. (**A**) Amino acid sequence of caledonicin with cut site distinguishing leader (bold) and core peptide identified (**B**) Multiple sequence alignment (MSA) of caledonicin bacteriocin from S. *caledonicus* APC 4137 and all known class IIc bacteriocins. Most closely related bacteriocin to caledonicin based on pairwise alignment of core peptide was enterocinNKR-5-3B (39.1%) (blue boxes) (**C**) Operon alignment of caledonicin identified from APC 4137 with enterocin-NKR-53B operon (Genbank accession: LC068607.1), and pairwise alignment of caledonicin and enterocin-NKR-5-3B with conservation parameters set to 80%. (**D**) Antimicrobial activity from partial purification elution in 70% IPA, 0.1% TFA against *L. lactis* subsp. *cremoris* HP and corresponding MALDI TOF mass spectrometry profile of 6,077.18 Da, where predicted mass calculated as 6,077.1 Da (Na+ adduct ion = 6,098.56Da; K+ adduct ion = 6,115.06 Da and potential oxidised peptide = 6,093.77Da). (**E**) Predicted structure of caledonicin as determined by Collabfold and viewed with PyMol v3.0 with extension to colour based on confidence where dark blue regions indicate high confidence (pLDDT > 90).

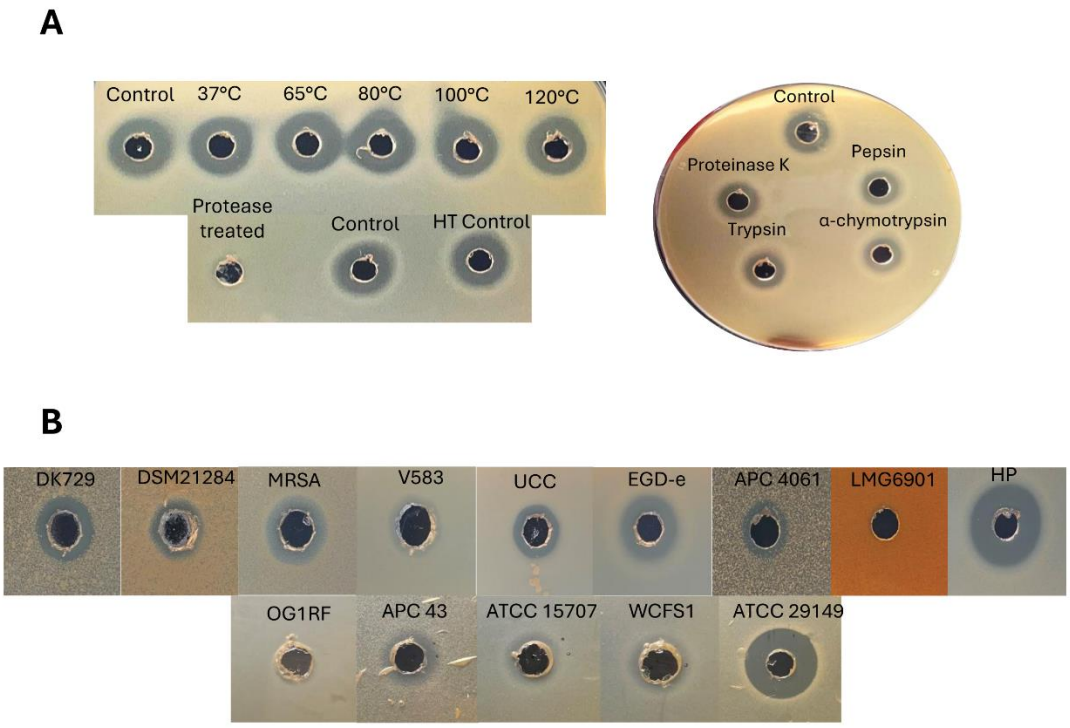
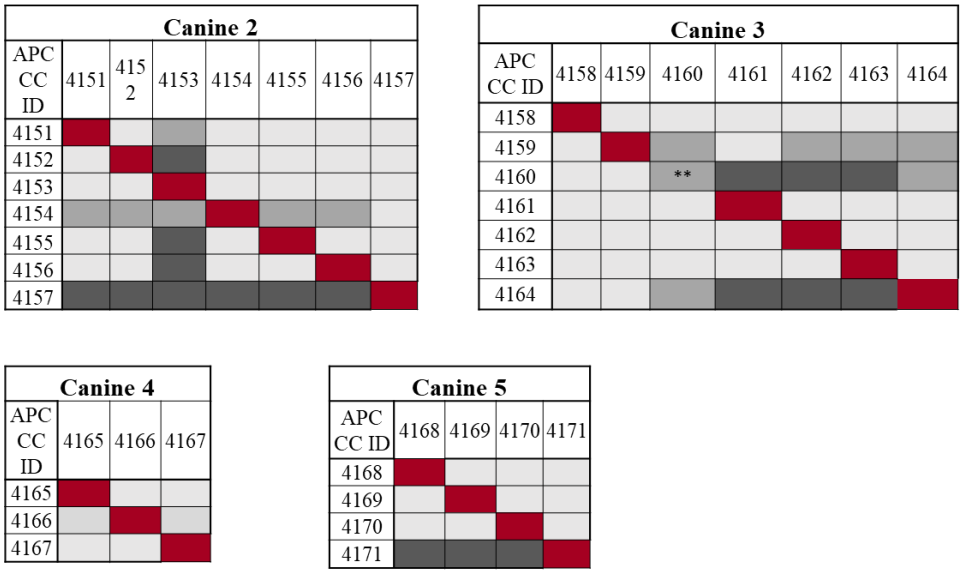


Figure 8. (A) Left: Heat stability (top) of 70% IPA, 0.1% TFA caledonicin fraction, and protease degradation (bottom) of the same fraction following treatment with proteinase K at 20 mg/mL to demonstrate the antimicrobial's proteinaceous nature. Right: Protease sensitivity assay on 70% IPA, 0.1% TFA caledonicin fraction following protease treatment with proteinase K, pepsin, trypsin, and α-chymotrypsin at 100 µg/mL. **(B)** Spectrum of inhibition assays via WDA against Gram positive indicators, including human gut related SIHUMI strains. Prior to carrying out this assay isopropanol from the 70% IPA, 0.1% TFA fraction was evaporated to concentrate the antimicrobial. Genus/species of strains listed in Figure 8B are as follows: ***S. pseudintermedius* DK729, *S. pseudintermedius* DSM 21284, MRSA DPC5645,** *E. faecalis* V583, ***L. innocua* UCC, *L. monocytogenes* EGD-e, *M. luteus* APC 4061**, *L. delbrueckii* subsp. *bulgaricus* LMG6901, ***L. lactis* subsp. *cremoris* HP.** SIHUMI: *E. faecalis* OG1RF, *C. difficile* APC 43, *B. longum* ATCC 15707, L. *plantarum* WCFS1, and ***M. gnavus* ATCC 29149.** Strains listed in bold are where strong zones of inhibition were observed in the assay.



Supplementary.

Figure S1. cross immunity of antimicrobial producing isolates from four of five canines in this study using overlay assay.

Chapter 5. Paenicidin L is a novel lantibiotic produced by *Paenibacillus* sp. APC 4171 with activity against pathogens.



Lacey

Abstract

This brief chapter will focus on the *Paenibacillus* bacterial isolate (APC 4171) which was first isolated in the canine screening study (chapter 4) of this thesis. This *Paenibacillus* strain belongs to a novel species of *Paenibacillus,* based on comparative genomics. The novel lanthipeptide produced by this isolate, termed paenicidin L, was also purified and characterised in this study. The peptide was purified using HPLC and it was determined that paenicidin L is a lantibiotic derived from a 57-amino acid propeptide (leader and core) with a predicted five- or six-ring structure in the mature peptide. It is protease-sensitive and heat-stable and exhibits antimicrobial activity against a range of bacteria including *Listeria monocytogenes*, *Listeria innocua*, *Enterococcus faecalis* and *Mediterraneibacter gnavus*.

Introduction

Bacteriocins are ribosomally synthesised antimicrobial peptides which have been described as alternatives to traditional antibiotics in an era of widespread antimicrobial resistance (AMR) (Sugrue, Ross and Hill, 2024).

One group of these bacteriocins are the class Ia lanthipeptides, so named due to the presence of (methyl)lanthionine rings. These rings are formed as a result of post-translational modifications (PTMs) of a ribosomally synthesised peptide, including dehydration of threonine and serine residues, and subsequent cyclisation of these dehydrated amino acids with cysteine residues for ring formation.

Paenibacillus bacteria are widely known for their potential use in agricultural settings as plant growth-promoting bacteria (Li *et al*., 2021), as well as the abundance of antimicrobials and secondary metabolites they produce (Grady *et al.*, 2016). These antimicrobials include bacteriocins, and more specifically lanthipeptides. To date, six lanthipeptides have been identified and characterised from *Paenibacillus* species (Lohans *et al.*, 2012, 2014; Teng *et al.*, 2012; Huang and Yousef, 2015; Baindara *et al.*, 2016; Park *et al.*, 2017). However, the *Paenibacillus* genus has been found to encode a diverse repertoire of lanthipeptides with a recent study reporting 221 lanthipeptide gene clusters identified across 127 strains of this bacteria, of which 150 were of the subclass I (LanBC modified) lanthipeptides (Baindara, Nayudu and Korpole, 2020). This large abundance of just one class of bacteriocins found within this genus would suggest this bacterium warrants further investigation for purification and characterisation of antimicrobial metabolites for future clinical or industrial applications.

Based on this we set out to purify and characterise a novel lanthipeptide identified within *Paenibacillus* APC 4171, a bacterium isolated from a canine ear in chapter 4 of this thesis. First, we determined this strain did indeed belong to a novel species *via* comparative genomics, *Paenibacillus polymyxa*, which was followed by purification and partial characterisation of one

of the lanthipeptides present in the genome, that we term paenicidin L. Paenicidin L was determined to be a protease-sensitive, heat-stable lanthipeptide. While mass spectrometry results did not completely agree with the mass predicted for a fully modified peptide, we suggest that the peptide contains five or six (methyl)lanthionine rings, however, further studies will need to be conducted to confirm this.

Materials and Methods

Identification of three novel bacteriocin gene clusters in *Paenibacillus* sp. APC 4171 genome *via in silico* analysis

Identification of lanthipeptides, including paenicidin L, and lasso peptide from *Paenibacillus* sp. APC 4171 is described in chapter 4 of this thesis.

Phylogenetic analysis of *Paenibacillus polymyxa* and APC 4171

Based on average nucleotide identity (ANI) taxonomy of the whole genome sequencing results in chapter 4, bacterial isolate APC 4171 was classified as *Paenibacillus polymyxa*. However, following *in silico* mining of this genome for bacteriocin and secondary metabolite production (described in methods of Chapter 4), and identification of the paenicidin-like lanthipeptide, the pro-peptide sequence of this lanthipeptide was run through the protein Basic Local Alignment Search Tool (BLASTp). Results of this search gave a 100% identity hit to the lanthipeptide previously identified in a novel *Paenibacillus* sp. (strain S25) (Li *et al*., 2021), herein termed paenicidin L by our group, however, this bacteriocin has not been characterised to date. Instead, this study by Li and co-workers (Li *et al*., 2021) characterised two *Paenibacillus* strains (S02 and S25) isolated from perennial ryegrass seeds, and *via* comparative genomics of 44 *P. polymyxa* genomes demonstrated that these *Paenibacillus* isolates belonged to a novel species based on their dendrogram-heatmap. Based on this novel species classification and the identical antimicrobial and secondary metabolite profile observed between APC 4171 and *Paenibacillus* sp. S25 phylogenetic analysis of APC 4171 was carried out with all *Paenibacillus polymyxa* genomes (n=112) available on the National Center for Biotechnology Information (NCBI) database (accessed 23 April 2024). Briefly, gene core alignment of all genomes was employed on Galaxy 24.1.rc1, followed by a phylogenetic tree created with interactive Tree of Life (iTOL) v 6.9 to determine the most closely related strain to APC 4171.

Purification of novel paenicidin L from *Paenibacillus* sp. APC 4171

Purification of the paenicidin L lanthipeptide from APC 4171 was carried out as per a previously employed method (Smith *et al.*, 2016), with modifications. Briefly, an overnight culture of APC 4171 strain was inoculated at 0.5% into separate 900 ml portions of purified BHI broth and incubated at 37 °C overnight. Following incubation, the cultures were centrifuged at 7000 × *g* at 4°C for 15 min. The supernatant was passed through a column containing ~70 g Amberlite XAD-16 beads and subsequently washed with 500 ml of 30% ethanol. The lanthipeptide was eluted from the column using 70% isopropanol containing 0.1% TFA (IPA-TFA). Simultaneously, bacterial cell pellets were resuspended in 200 ml 70% IPA-0.1% TFA and stirred at room temperature for 1.5 h. This cell suspension was then centrifuged at 5,000 × *g* at 4 °C for 20 min, and the supernatant was retained. The column eluant was pooled with the post-centrifugation supernatant and isopropanol evaporated using a rotary evaporator (Rotavapor R-205; Büchi, Switzerland). The sample was subsequently passed through a 10-g (60-ml) Strata C_{18} E column (Phenomenex) pre-equilibrated with 60 ml methanol (Fisher Scientific, UK) and 60 ml HPLC-grade H_2O. After 120 ml of 20% ethanol was applied, the lanthipeptide was eluted from the column using 60 ml of 70% isopropanol–0.1% TFA. For HPLC purification, 12-ml volumes were concentrated to a volume of 2 ml by rotary evaporation and applied to a Phenomenex C_{12} reverse-phase HPLC column (Jupiter 4-μm Proteo, 90 Å, 250 mm by 10.0 mm, 4 μm) previously equilibrated with 25% acetonitrile–0.1% TFA. The lanthipeptide was eluted via a gradient of 25 to 70% acetonitrile–0.1% TFA that was developed from 10 to 40 min at a flow rate of 3.2 ml min^{-1}. Lanthipeptide-containing fractions were pooled and assessed for presence of the paenicidin-like lanthipeptide via MALDI-TOF mass spectrometry for the peptide's predicted mass.

Antimicrobial activity spectrum of paenicidin L

Following confirmation of activity of the purified paenicidin L against *L. lactis* HP, the spectrum of inhibition was determined via well diffusion assay (WDA) as previously described against gram-positive indicators used in chapter 4 including those which make up a Simplified Human Intestinal Microbiota (SIHUMI) (Ríos Colombo *et al.*, 2023) with the addition of a *Clostridioides difficile* indicator (Table 1A and B).

Protease and heat stability of paenicidin L

The heat stability and protease sensitivity of paenicidin L were determined by WDA on the HPLC fractions. To investigate if the antimicrobial produced was proteinaceous in nature, the eluent was subjected to protease treatment with Proteinase K (Sigma) to a final concentration of 20 mg mL^{-1} at 37 °C for 3 hr before heat deactivating the protease enzyme at 95 °C for 5 mins and testing for antimicrobial activity via WDA. Controls for this test included untreated HPLC fraction (diluted to the same concentration as test fraction).

The HPLC fractions 46 and 47 were incubated for 15 min at a range of temperatures, 65 °C, 80 °C, 100 °C, and 120 °C, before performing a WDA in 1.5% M17 agar supplemented with 0.5% glucose seeded with 0.25% of *L. lactis* HP. Untreated HPLC fraction was used as a control.

MALDI-TOF mass spectrometry

MALDI TOF mass spectrometry was performed on the HPLC fractions exhibiting antimicrobial activity from paenicidin L purification using an iDPlus Performance MALDI TOF mass spectrometer (Shimadzu Europa GmbH , Duisberg, Germany). An aliquot (0.5 µL) of matrix solution (α-cyano 4-hydroxy cinnamic acid, 10 mg mL^{-1} in acetonitrile-0.1% (v/v) trifluoroacetic acid) was deposited onto the target and left for 20 s before being removed. The

residual solution was allowed to air-dry and 0.5 µL of the sample solution was deposited onto the pre-coated sample spot; 0.5 µL of matrix solution was added to the deposited sample and allowed to air-dry. Samples were then analysed in positive-ion linear mode.

Results

Paenibacillus sp. APC 4171 genome encodes three putative novel bacteriocins.

Genome analysis of a *Paenibacillus* strain, APC 4171, isolated from a canine ear revealed the presence of three putative novel bacteriocin gene clusters. These encode a lasso peptide with 45.24% identity to a previously characterised lasso peptide, paeninodin, isolated from *Paenibacillus dendritiformis* C454 (Zhu *et al.*, 2016), a lanthipeptide gene cluster encoding a novel variant of paenilan (94% identity) first isolated from *Paenibacillus polymyxa* E681 (Park *et al.*, 2017), and a second lanthipeptide gene cluster annotated as paenicidin B (Lohans *et al.*, 2014) by antiSMASH7.0. Based on pairwise alignment of the amino acid sequences of the structural peptide (leader and core) of the paenicidin B-like lanthipeptide it was calculated to have 47.46% and 44.07% identity to paenicidin B (Lohans *et al.*, 2014)and paenicidin A (Lohans *et al.*, 2012), respectively. The antimicrobial polypeptide polymyxin, which exhibits strong activity against gram-negative bacteria (Mohapatra, Dwibedy and Padhy, 2021), was also identified within the genome of APC 4171. Based on secondary metabolite analysis *via* antiSMASH7.0 of another *Paenibacillus* genome submitted to the NCBI databank, all three of the bacteriocin gene clusters have previously been identified in a proposed novel *Paenibacillus* species; *Paenibacillus* sp. S25, which was isolated from perennial ryegrass (Li *et al.*, 2021). Strain S25 contains the same three bacteriocin gene clusters with 100% amino acid identity to the structural peptides found in APC 4171. While we report the amino acid percentage identity of the structural peptides based on pairwise alignment in chapter 4 of this thesis (conducted in Jalview version 2.11.3.2), Li and co-workers reported the percentage identity based on the whole gene cluster of each bacteriocin (Li *et al*., 2021-supplementary Table 8). Here, we name this novel lanthipeptide as paenicidin L. Multiple sequence alignment (MSA) of propeptides identified within *Paenibacillus* sp. APC 4171 and the most closely related peptides, including operon alignments, are presented in Chapter 4, Figures 5 of this thesis.

Phylogenetic analysis of APC 4171 and *Paenibacillus polymyxa* genomes

Phylogenetic analysis of APC 4171 with 112 *P. polymyxa* genomes available on NCBI via core gene alignment and the subsequent construction of a phylogenetic tree (Figure 1) demonstrated that APC 4171 was most closely related to *Paenibacillus* sp. S25 mentioned above (Li *et al*., 2021). Based on this and the result reported by Li and co-workers in their study, we suggest that APC 4171 belongs to a novel species of *Paenibacillus*.

HPLC peptide purification

HPLC performed on antimicrobial purified from *Paenibacillus* sp. APC 4171 resulted in one large symmetrical peak of approximately 1000 mV that eluted from the C_{12} column at ~70% acetonitrile between 39-40 minutes (Figure 2A). This large peak was distributed across two fractions (#46 and #47), however, all fractions obtained throughout the HPLC run were tested against *M. luteus* APC 4061, *E. coli* MG1655 and *L. lactis* HP (data not shown). Antimicrobial activity was only observed in fractions #46 and #47 (Figure 2A)

Paenilan has previously been reported to exhibit activity against *M. luteus* (Park *et al.*, 2017), while polymyxin is a polypeptide known for its inhibition of gram-negative bacteria (Mohapatra, Dwibedy and Padhy, 2021). The purified fractions #46 and #47 exhibited no activity against *M. luteus* or *E. coli* (data not shown) but gave potent activity against *L. lactis* HP (Figure 2A), suggesting the paenilan lanthipeptide and polymyxin were not purified in this study. Subsequent mass spectrometry of fraction #47 also confirmed this absence of these (discussed below).

Protease and heat stability of paenicidin L

Protease treatments of HPLC fractions #46 and #47 revealed the antimicrobial present to be proteinaceous in nature as it was partially degraded by proteinase K at a high concentration of 20 mg mL^{-1} compared to untreated controls (Figure 2B). Interestingly, residual activity was still observed against *L. lactis* HP despite treatment for several hours in the presence of such a high concentration of protease enzyme. This result further suggests a highly modified bacteriocin, such as a lanthipeptide, is present in the HPLC fractions. As expected for a lanthipeptide the activity was stable at all temperatures tested, including close to autoclave conditions at 120 °C (Figure 2C).

Mass spectrometry of paenicidin L

The predicted mass of paenicidin L was calculated as 3317 Da based on a predicted six-ring structure due to the presence of six cysteine residues within the core peptide sequence. This six-ring structure was also reported for paenicidin A and B (Lohans *et al.*, 2012, 2014) with no further modifications, despite the presence of a number of threonine and serine residues in each of these peptides that are often dehydrated in lanthipeptides. However, the mass spectrometry profile observed for fraction #47 includes three peaks of 3138 Da, 3177 Da and 3160 Da (Figure 2D). Based on these observed masses further inspection and calculation of potential masses were carried out. We propose that the 3138 Da mass corresponds to a modified peptide with the first two amino acid residues (valine and threonine) cleaved off and with only five lanthionine rings. We have arbitrarily chosen to eliminate the first ring in our depiction in Figure 3, but it could theoretically be any one of the six lanthionine rings that are not formed. The masses 3160 Da and 3177 Da we believe to be the same peptide with sodium (+22 Da) and potassium (+38) adduct ions, respectively. The basis for predicting the absence of the first ring is due to data obtained with another lanthipeptide identified and partially

purified from a *Paenibacillus* strain from our group which is currently unpublished, but similar observations have been made with this peptide mass spectrometry profile (Ivan Sugrue- personal communication).

Antimicrobial activity of paenicidin L against drug-resistant pathogens

The antimicrobial activity of paenicidin L HPLC fractions #46 and #47 was assayed *via* WDA against selected indicators of interest. Initial WDAs were carried out on *L. lactis* HP, *M. luteus* APC 4061 and *E. coli* MG1655 (data not shown) as described above. This was to confirm that the HPLC fraction was not the paenilan lanthipeptide or polymyxin both of which are also encoded on the genome of APC 4171. The purified fractions #46 and #47 exhibited no activity against *M. luteus* (data not shown) but gave potent activity against *L. lactis* HP (Figure 2A), suggesting the paenilan lanthipeptide was not purified in this study. Mass spectrometry of fraction #47 also confirmed this absence of paenilan, which has a mass of 2510.1 Da (Park *et al.*, 2017) (Figure 2D). Additional inhibition activity of fractions #46 and #47 was tested against the indicators listed in Tables 1A and 1B. These assays show that paenicidin L exhibits activity against *Listeria monocytogenes* EGD-e, *Enterococcus faecalis* (VRE) V583, and *Enterococcus faecalis* OG1RF. However, the most sensitive indicators to this peptide were found to be *L. lactis* HP, *L. innocua* UCC and *Mediterraneibacter gnavus* ATCC 29149 (Figure 2E).

Discussion

AMR is an ongoing issue within human and animal settings. It has been reported that novel antimicrobials to replace or be used in combination with traditional antibiotics will aid in the fight against AMR pathogens (O'Neill, 2019). One such alternative to help tackle this issue is bacteriocins. Bacteriocins are ribosomally synthesised antimicrobial peptides produced by bacteria that inhibit the growth of other bacteria (Cotter, Hill and R.Paul Ross, 2005). Bacteriocins are divided into two separate classes based on the presence (class I) and absence (class II) of modifications (Pérez-Ramos *et al.*, 2021; Soltani *et al.*, 2021; Sugrue, Ross and Hill, 2024). One subclass of the class I bacteriocins are the lanthipeptides, so-called due to the presence of their unusual lanthionine and methyl-lanthionine ring structures. Lanthipeptides are one of the most widely studied classes of bacteriocins, in particular nisin which has been researched for nearly a century and is currently approved for use as a food preservative (E234) (Field *et al.*, 2023), as well as several nisin based products used in animal settings for treatment of mastitis in dairy cows, for example Preva medicated wipes by Bayer (Field *et al.*, 2015).

Paenibacillus bacteria have been isolated from many different environmental niches including humans, animals and in particular plants where they are considered a growth-promoting bacteria in crops (Grady *et al*., 2016; Li *et al*., 2021). *Paenibacillus* can produce a plethora of secondary metabolites including antimicrobials such as tridecaptin, fusaricidin, polys and bacteriocins (Grady *et al*., 2016).There is a high abundance of lanthipeptide gene clusters in *Paenibacillus* genomes which have been identified *via in silico* analysis (Baindara, Nayudu and Korpole, 2020), with some purified and characterised that exhibit broad spectrums of activity (Lohans *et al.*, 2012, 2014; Teng *et al.*, 2012; Huang and Yousef, 2015; Baindara *et al*., 2016; Park *et al.*, 2017).

Here we describe the identification of a novel lanthipeptide, that we have termed paenicidin L. We were able to purify the peptide by HPLC into a single symmetrical peak that showed activity (Figure 2A). The active HPLC fractions do not target *M. luteus* (data not shown), a property that has been reported for the lanthipeptide, paenilan (Park *et al.*, 2017), that is also encoded in the genome of *Paenibacillus* sp. APC 4171, therefore based on this lack of inhibition against *M. luteus* and mass spectrometry results (Figure 2D) it can be confirmed that paenilan is not present in the HPLC fractions in this study. In addition, the physicochemical properties of the active fractions indicate a protease-degraded, heat-stable antimicrobial has been purified *via* this protocol (Figure 2B and 2C).

Based on these results we propose that paenicidin L is a novel lanthipeptide. Due to the fact that the mass spectrometry (3138 Da) does not match the predicted mass for a six-ring lanthipeptide (3318 Da) further analysis is required. Thus far, based on the mass spectrometry results we predict that the first two amino acids (VT) of the peptide have been removed and that one (methyl)lanthionine ring is missing which we summarise in Figure 3. While we arbitrarily chose to eliminate the first ring in the lanthipeptide in our depiction (Figure 3), we acknowledge this unmodified ring structure could be applied to any of the six rings within the peptide. Further assays will need to be carried out to determine the actual structure of the lanthipeptide. Such assays will include further purification of the peptide followed by nuclear magnetic resonance (NMR) or N-terminal amino acid sequencing to determine the chemical structure of the lanthipeptide, which is currently in progress.

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Tables and Figures

Table 1. Bacterial indicator strains and their growth conditions used in this study.

A: Micro-organism	Conditions
Lactococcus lactis subsp. *cremoris* HP	GM17, aerobic, 30 ˚C
Micrococcus luteus APC 4061	BHI, aerobic, 37 ˚C
Staphylococcus pseudintermedius DK 729	BHI, aerobic, 37 ˚C
Staphylococcus pseudintermedius DSM 21284	BHI, aerobic, 37 ˚C
Listeria innocua UCC	BHI, aerobic, 37 ˚C
Listeria monocytogenes EGDe	BHI, aerobic, 37 ˚C
MRSA DPC 5645	BHI, aerobic, 37 ˚C
Enterococcus faecalis VRE V583	BHI, aerobic, 37 ˚C
Cronobacter sakazakii DPC 6440	LB, aerobic/anaerobic, 37 ˚C
Salmonella enterica serovar typhymurium UK1	LB, anaerobic/aerobic, 37 ˚C
Klebsiella pneumoniae NCIMB 13218	LB, anaerobic/aerobic, 37 ˚C
Escherichia coli ETEC K88f4	LB, anaerobic/aerobic, 37 ˚C
Escherichia coli ETEC F18ab	LB, anaerobic/aerobic, 37 ˚C
Escherichia coli K12 MG1655 (ATCC 47076)	LB, Anaerobic/aerobic, 37 ˚C
Pseudomonas aeruginosa PA01	BHI, aerobic, 30 ˚C
SIHUMI strains	
B: Species/Strain	Conditions
Enterococcus faecalis OG1RF	LYHBHI, anaerobic, 37 ˚C
Lactiplantibacillus plantarum WCFS1 (NCIMB 8826)	LYHBHI, anaerobic, 37 ˚C
Bifidobacterium longum ATCC 15707	LYHBHI, anaerobic, 37 ˚C
Mediterraneibacter gnavus ATCC 29149	LYHBHI, anaerobic, 37 ˚C

Table 1. continued

Clostridioides difficile APC 43 (ATCC 43255)	LYHBHI, anaerobic, 37 ˚C

ATCC, American Type Culture Collection; APC, APC Microbiome Ireland Culture Collection; DPC, Teagasc Culture Collection; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; DSM, Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures; UCC, University College Cork, Cork, Ireland; NCIMB, National Collection of Industrial, Food and Marine Bacteria. LB; Luria-Bertani, BHI; Brain Heart Infusion, LYHBHI; BHI medium supplemented with 0.5% yeast extract (Difco), 5 mg/liter hemin, cellobiose (1 mg/ml; Sigma–Aldrich), maltose (1 mg/ml; Sigma), and cysteine (0.5 mg/ml; Sigma), GM17; M17 supplemented with 0.5% glucose.

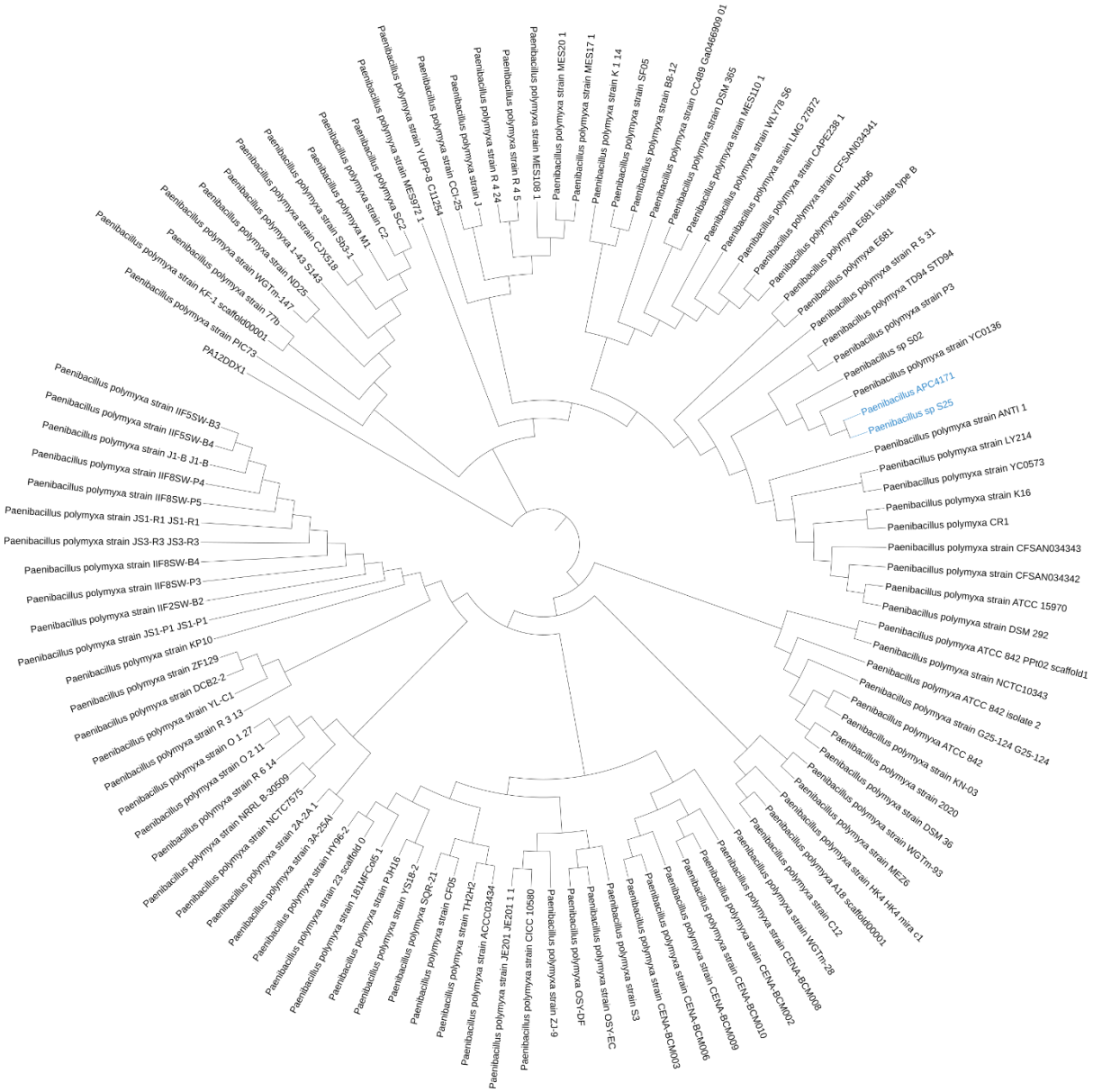


Figure 1. Phylogenetic tree of 112 *Paenibacillus polymyxa* strains accessed from NCBI (April 2024) aligned with *Paenibacillus* sp. S25, S02 (Li *et al.*, 2021) and APC 4171. *Paenibacillus* sp. APC 4171 and S25 are highlighted in blue.

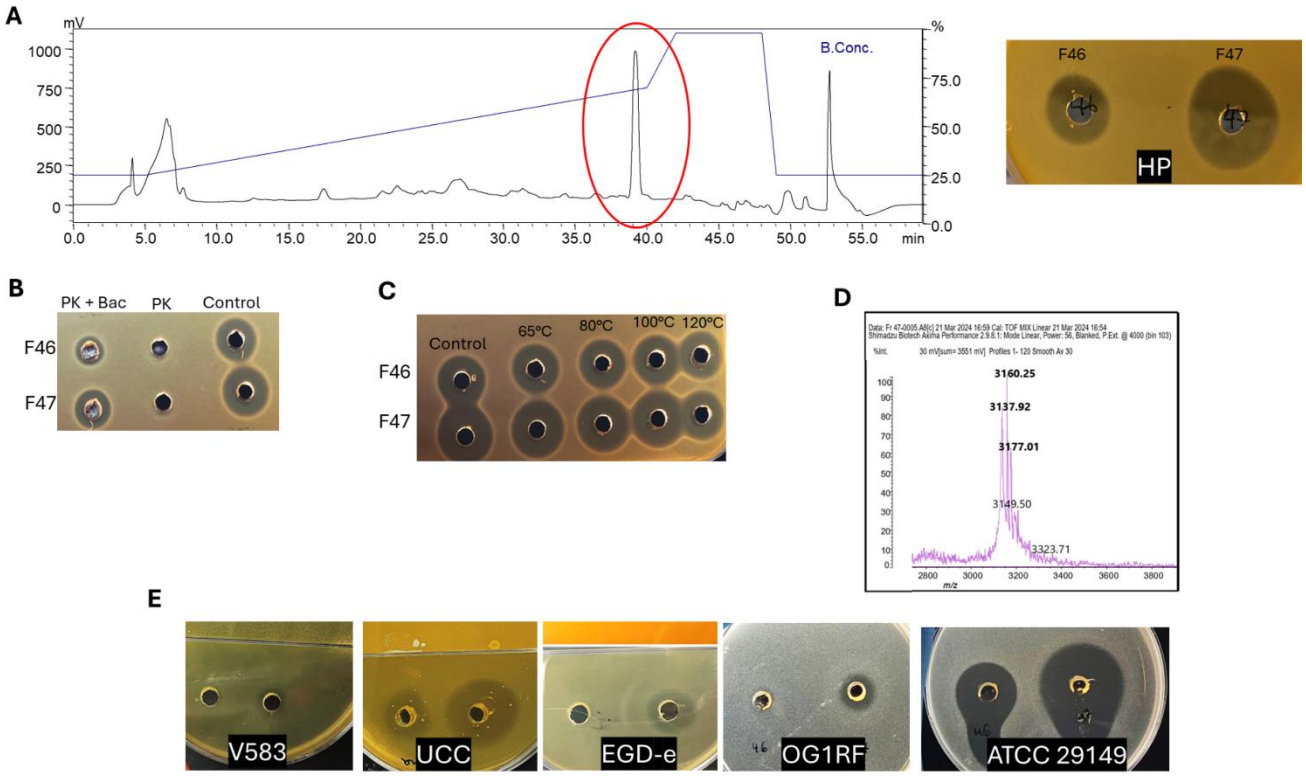


Figure 2. (A) HPLC profile of lanthipeptide purification from *Paenibacillus* sp. APC 4171. The peak containing activity is circled in red with corresponding antimicrobial activity of fractions #46 (left) and #47 (right) against *L. lactis* HP. **(B)** Protease sensitivity of fractions #46 and #47. **(**PK + Bac denotes bacteriocin treated with proteinase K at 20 mg mL^{-1}; PK denotes proteinase K at the same concentration (20 mg mL^{-1}) and Control is bacteriocin diluted to the same volume as PK + Bac test.) **(C)** Heat stability of antimicrobials fractions #46 and #47. **(D)** Mass spectrometry profile of HPLC fraction #47. **(E)** Indicator organisms that are sensitive to the antimicrobial peptide from HPLC fractions #46 (left) and #47 (right). (Genus/species of indicators exhibiting sensitivity to paenicidin L are as follows: *E. faeclis* V583 (VRE), *L. innocua* UCC, *Li. monocytogenes* EGD-e, *E. faecalis* OG1RF and *M. gnavus* ATCC 29149)

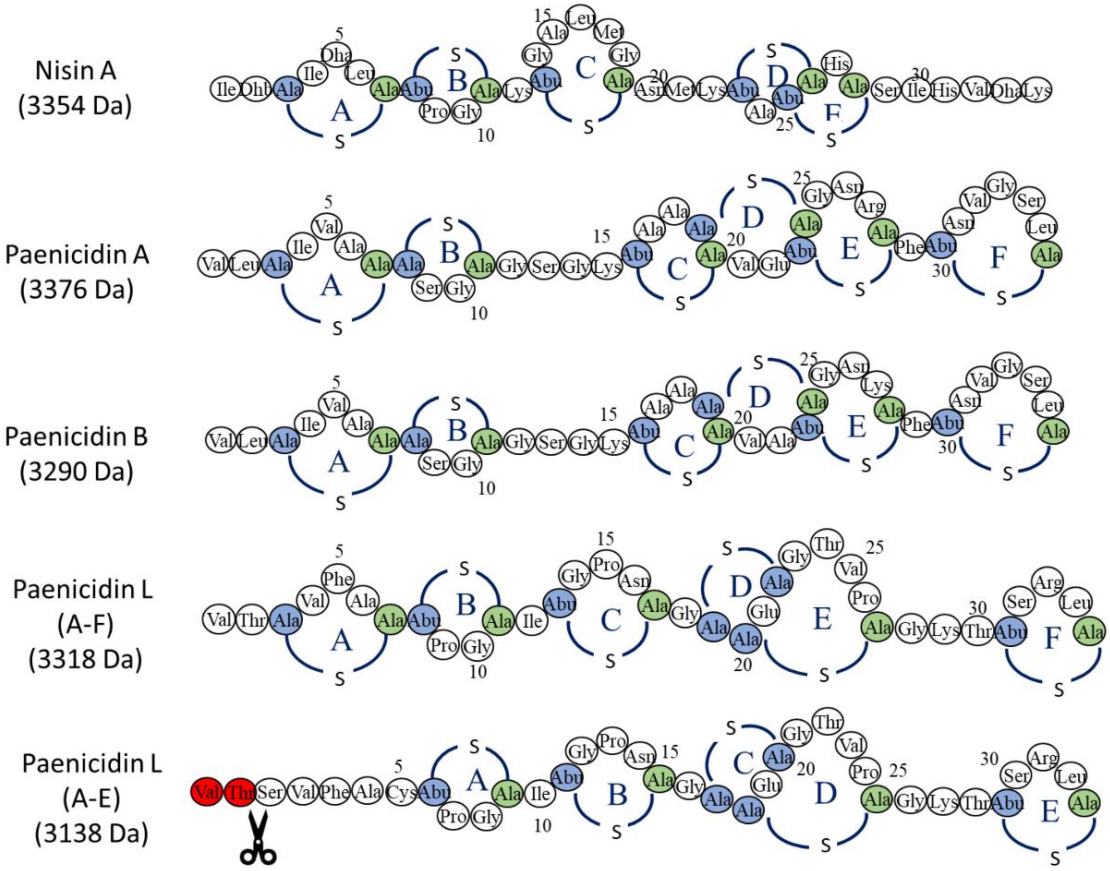


Figure 3. Predicted ring formation of paenicidin L in comparison to nisin A, paenicidin A and paenicidin B. Blue residues denote threonine/serine residues which are dehydrated and form rings with cysteines which are coloured green. Paenicidin L (A-F) demonstrates six dehydrations of threonine/serine residues with six subsequent rings formed. Paenicidin L (A-E) demonstrates the cleavage of the first two residues (Val and Thr in red) and one ring not formed in this peptide based on mass spectrometry profile observed.

Thesis Discussion

Bacteriocins have been described as potential alternatives to traditional antibiotics in the AMR era. One subclass of bacteriocins are the highly researched lantibiotics, of which nisin is the most investigated. Although lantibiotics have been well studied for their potent antimicrobial activity, other genes and corresponding proteins within the biosynthetic gene clusters have been documented and characterised also. Of these genes are the lantibiotic protection mechanisms or immunity proteins, termed LanI and LanFEG for lantibiotics. Nisin and many lantibiotic have been shown to exhibit potential as alternatives to treat multi-drug resistant (MDR) pathogens. However, there have been cases of lantibiotic resistance reported. As nisin is described as the representative lantibiotic of this class, it is not surprising that much research in relation to lantibiotic resistance focuses on the specific nisin resistance protein Nsr and NsrFP. This research includes structural modelling of these resistance proteins to understand specific interactions with the antimicrobial peptide. Chapter 1 of this thesis reviews and discusses lantibiotic immunity and resistance with a particular focus on the lantibiotic specific resistance proteins, including the BceAB-type and Cpr-type ABC transporters. This chapter also discusses the potential for bioengineering of these lantibiotics to overcome lantibiotic resistance. While bioengineering of these proteins has been carried out to overcome MDR pathogens, little research has been conducted to produce mutants with an aim to overcome the lantibiotic resistance mechanisms discussed in this chapter. Combinatorial therapies are also discussed as a potential approach to overcome lantibiotic resistance.

Nisin, the most widely studied bacteriocin of the subclass 1a lanthipeptides has been investigated for its antimicrobial activity against multi-drug resistant pathogens. The operon of nisin, however, also contains genes for the immunity system (NisI/F/E/G) that protects the nisin producing strain from the antimicrobial action of the peptide, and the lanthipeptide regulation system (nisRK) which controls its production.

In line with the current knowledge on lantibiotic immunity and resistance discussed in chapter 1, this thesis investigated the immunity and resistance machinery of the most widely studied lantibiotic, nisin. In chapter 2 recombinant DNA of the *nisI*, n*isFEG*, *nsr* and *nsrFP* genes was transformed into three *Lactococcus lactis* strains, MG1614, NZ9800 and NZ9700, all of which are from the same genetic background. Genes were cloned into the pNZ44 plasmid, containing the putative P44 lactococcal promoter. The first aim of this study was to determine which of the nisin protection mechanisms conferred the most protection to the nisin sensitive train MG1614. Results observed showed the protein which confers the most protection to nisin in a sensitive strain is both nisin immunity proteins when expressed together (NisIFEG), suggesting a co-operative system as has been previously suggested. The nisin resistance ABC transporter, NsrFP, offered the same level of protection as the NisIFEG proteins expressed together with both reporting an MIC of 1.875µM. It was also determined what impact increasing the number of copies of nisin resistance proteins in MG1614 already containing the natural nisin resistance plasmid, pNP40, or increasing the number of copies of immunity proteins in an already immune strain NZ9800 has on nisin sensitivity. Results demonstrated it was possible to increase nisin resistance via increased copies of the *nsr* gene on the pNZ44 plasmid with pNP40 also present in the MG1614 strain; however, implementation of the *nsrFP* gene on the plasmid decreased the resistance to the same level found within natural nisin resistant *Streptococcus agalactiae* strains previously described to contain the entire nisin resistance operon (NsrR, NsrK, Nsr and NsrFP) (Hayes *et al.*, 2019). For NZ9800 increasing nisin immunity was achievable with either of the constructs present (pNZ44*nisI* or pNZ44*nisFEG*) in the strain, however, not to the same level of protection exhibited by the nisin producing strain NZ9700 which protects itself from nisin up to 15µM concentrations. This suggested the nature designed system in a nisin producing strain offers the optimal protection to the lanthipeptide. Finally, this study determined the effect of

increasing immunity in a nisin producing strain on the lanthipeptide production. Results proved the implementation of the NisI protein increased the protection of the strain two-fold compared to the control, NZ9700, while increased copies of NisFEG decreased the immunity to half that of the control. While implementation of all immunity genes separately (*nisI, nisFEG*) and when co-expressed (*nisIFEG*) did not positively or negatively affect the growth of the strain, the production of nisin was not increased by the presence of these constructs. In contrast, the NZ9700 pNZ44*nisFEG* strain was found to produce no nisin based on the WDA and area under the curve assays performed in this study. We hypothesise this decrease in production was as a result of increased copies of the ABC transporter protein exporting nisin away from the cell membrane, rendering the strain incapable of inducing its own lantibiotic production via the two-component system, NisRK. Further work that could prove this hypothesis would be a nisin induction reporter assay, for example GFP under the control of the P*nisA* or P*nisF* promoters. This has been previously employed as an assay to test nisin induction capability (Field *et al.*, 2019; O'Connor *et al.*, 2020). If a plasmid which produces GFP under the control of the P*nisA* or P*nisF* nisin-controlled promoters was introduced into NZ9700 strains with and without pNZ44 constructs and fluorescence measured, we expect to observe a significantly less amount of fluorescence from the NZ9700 pNZ44*nisFEG* strain. These assays concluded that while increased immunity does not impact growth rate or overall cell growth based on OD, it also does not positively impact nisin production. Other future work that could be carried out on this could be bioengineering of the nisin propeptide to gain a better understanding of these protection proteins activity on the antimicrobial peptide, and to produce variants which can overcome these resistance mechanisms in the AMR crisis.

Numerous studies have been carried out on bioengineering nisin at various locations within the peptide to date. For example, studies have been conducted on bioengineering the nisin structural gene to produce variants with enhanced stability in certain environments (Field *et*

al., 2019), enhanced activity against clinically relevant pathogens (Field *et al*., 2015; Twomey *et al*., 2020), including Gram negative pathogens (Li, Montalban-Lopez and Kuipers, 2018), or reduced activity with retained induction capability for protein expression applications (O'Connor *et al.*, 2020). However, there are limited studies investigating overcoming the immunity/resistance mechanisms. One such study includes the natural nisin variant nisin H and its bioengineered derivative (F1I) where this F1I variant exhibited greater antimicrobial activity against NisI, NisFEG, Nsr and NsrFP in comparison to wildtype peptide (Reiners *et al.*, 2020). This result warrants further investigation of bioengineered nisin derivates against these immunity and resistance proteins in order to overcome future nisin resistance development.

As mentioned previously, another characteristic of lanthipeptides is the ability for the peptide to induce its own production, via the two-component regulation system, termed LanRK. Researchers have taken advantage of the nisin biosynthesis pathway, including NisRK and the nisin A promoter, *PnisA*, to create the NIsin Controlled gene Expression system (NICE). While this system is almost 30 years old and has been used in studies to induce the production of proteins of interest via induction from nisin (Mierau *et al.*, 2005; Esteban *et al.*, 2013; Szczepankowska *et al.*, 2017; Zhang *et al.*, 2018), one limiting factor of the system is the antimicrobial activity of nisin itself, and its effect on nisin-sensitive strains encoding the protein of interest, as has been shown for other protein expression *via* nisin induction assays (Reunanen and Saris, 2003; Zhang *et al.*, 2018). Based on this limitation, chapter 3 investigated the effect of a ring-B bioengineered nisin variant, termed nisin M, which retains full induction capacity while simultaneously displaying little antimicrobial activity against nisin sensitive strains. Nisin M was shown to display between a 4-16-fold decrease in activity against the strains tested based on MIC assays in comparison to wild-type nisin A. RLU readings of green fluorescent protein (GFP) in *L. lactis* NZ9000 containing the nisin inducible promoter, *PnisA*, and a GFP gene downstream of this promoter on pNZ8150

following induction by nisin M and A at varying concentrations was monitored. Results demonstrated the ability of nisin M to induce GFP expression up to 300 ng mL^{-1}, with optimal induction concentration determined to be 50 ng mL^{-1} based on RLU readings with no significant impact on growth of the strain even at the higher concentration tested, concluding nisin M as a nisin alternative to the wildtype peptide in induction of the NICE system; in particular for strains highly sensitive to nisin A.

As mentioned in chapter 4, AMR is a continuous risk to human and animal health, and bacteriocins have been described as alternatives to traditional antibiotics. Many screening studies with an aim to identify and characterise novel bacteriocins have been conducted from both human and animal sources. However, to our knowledge no such screening studies have been conducted from companion animals, in particular canines. Based on this being a novel niche for AMPs chapter 4 focuses on a screening study aimed to identify putative novel bacteriocins across four sites from five canines. Based on *in silico* and *in vitro* analysis of the antimicrobial potential of 8 bacterial isolates, a total of 14 putative novel bacteriocins were identified, one of which was a novel circular bacteriocin from a previously identified novel species, *Staphylococcus caledonicus* strain (APC 4137). This bacteriocin, termed caledonicin, is a 64 amino acid circular peptide with antimicrobial activity against pathogenic bacteria, including *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus pseudintermedius* (MRSP), and the IBD/IBS associated gut bacterium *Mediterraneibacter* (*Ruminococcus) gnavus* ATTC 29149. This study is the first conducted canine screen for novel bacteriocins, and the first identified novel bacteriocin from the *Staphylococcus caledonicus* species. Indeed, further characterisation of this antimicrobial is to be completed, including determination of all gene function within the caledonicin operon, via gene knockout methods.

The final chapter of this thesis characterises another lantibiotic isolated from a canine ear in chapter 4. This lantibiotic was found *via in silico* mining of a *Paenibacillus* isolates (APC 4171) which was determined to belong to a novel species of *Paenibacillus via* comparative genomics. The lantibiotic identified here was amongst a plethora of other secondary metabolites identified within the genome of this strain with antiSMASH, including a novel paenilan lantibiotic, a novel lasso peptide, polymyxin, fusaricidin and tridecaptin, to name a few. The lantibiotic characterised in chapter 5 was most closely related to paenicidin B with 47.5% identity across the prepeptide amino acid sequences. Following purification of this lantibiotic, termed paenicidin L via HPLC, mass spectrometry analysis revealed a mass of 3138 Da. This mass did not match our calculated mass of 3318 Da for the modified lantibiotic, however due to personal communications which report a lantibiotic from another *Paenibacillus* strain with similar observations in mass spectrometry profiles, we believe this lantibiotic contains five or six (methyl)lanthionine rings within its structure with the first two residues (valine and threonine) cleaved off. Of course, further analysis would need to be carried out to confirm this, such as N-terminal amino acid sequencing or NMR spectroscopy. Paenicidin L was determined to be a protease degraded, heat stable antimicrobial peptide which exhibits broad spectrum of activity.

In conclusion, this thesis covers the topics of lantibiotics and the evolution of resistance to these peptides, investigation of the nisin immunity and resistance mechanisms in sensitive and immune strains, including the effect these proteins have on nisin production. Finally, a screening study identified 14 putative novel bacteriocins, two of which are characterised here and exhibit activity against a wide range of indicators, including clinically relevant pathogens.

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"If you can dream it, you can do it"

-Walt Disney