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Identification and Creation of Novel Bacteriocins with Potential Food and Clinical Applications

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by
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Declaration

I declare that this thesis, which I submit to Munster Technological University, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme, this is duly acknowledged, such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in Munster Technological University (MTU) or elsewhere on the basis of this work.

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List of Abbreviations

ABC	ATP-binding Cassette
ACN	Acetonitrile
bp	Base Pairs
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
cBHI	Clarified BHI
CDC	Centres for Disease Control and Prevention
CFS	Cell-free supernatant
CFU	Colony Forming Units
CFU/ml	Colony Forming Units per millilitre
CMS	Colony mass spectrometry
CoNS	Coagulase-negative *Staphylococci*
CoPS	Coagulase-positive *Staphylococci*
CUH	Cork University Hospital
Da	Daltons
Dhb	Dehydrobutyrine
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
FDA	US Food and Drug Administration
FSC	Food Safety Criteria
GM17	M17 broth + 0.5% glucose
GRAS	Generally Recognized as Safe
HK	Histidine protein kinase
IPA	Isopropanol
kDa	Kilodaltons
LAB	Lactic Acid Bacteria
LB	Luria-Bertani broth
LSA	Listeria Selective Agar
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight Mass Spectrometric

MIC	Minimal Inhibitory Concentration
MRSA	Methicillin-Resistant *S. aureus*
MS	Mass Spectrometric
MSA	Mannitol Salt Agar
MTU	Munster Technological University
MW	Molecular Weight
NSR	Nisin Resistance Gene
NSR+	Contains Nisin Resistance Gene
NSR-	Lacks Nisin Resistance Gene
OD	Optical Density
ORF / *orf*	Open Reading Frames
PBS	Phosphate Buffered Saline
PI	Iso-electric point
PSM	Phenol Soluble Modulin
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
RR	Response Regulator
RTE	Ready-to-Eat
TFA	Trifluoroacetic Acid
TSB-YE	Tryptic Soy Broth (TSB) supplemented with Yeast Extract
TY	Tryptone Yeast
UCC	University College Cork
USDA	United States Department of Agriculture
VRE	Vancomycin-Resistant *Enterococci*
WCE	Whole Cell Extracts
WT	Wild-Type

Abbreviations of Amino Acids

Amino Acid	Three Letter Code	One Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Publications and Posters generated from this thesis

Peer-reviewed publications (see Appendices)

Lynch D, O'Connor PM, Cotter PD, Hill C, Field D, Begley M. (2019) Identification and characterisation of capidermicin, a novel bacteriocin produced by *Staphylococcus capitis*. Tse H, editor. PLoS One. 2019 Oct 16;14(10):e0223541

Lynch D, Hill C, Field D, Begley M. (2021) Inhibition of *Listeria monocytogenes* by the *Staphylococcus capitis* - derived bacteriocin capidermicin. 2020. Food Microbiology. 94, 103661. https://doi.org/10.1016/j.fm.2020.103661

Posters presentations

David Lynch, Colin Hill, Paul D. Cotter, Des Field, Máire Begley. Investigation of bacteriocin production by a bank of *Staphylococcus epidermidis* strains. (2016). SGM conference Exploring the microbe-immune system interface. Rochestown Park Hotel in Rochestown, Cork, Ireland. September 1st-2nd, 2016.

David Lynch, Paula O' Connor, Colin Hill, Paul D. Cotter, Des Field and Máire Begley. Identification and characterisation of capidermicin, a novel class II broad spectrum bacteriocin from *Staphylococcus capitis* (2018). AMP conference, Sixth International Symposium on Antimicrobial Peptides. Poitiers, France. June 6th-8th, 2018.

Thesis abstract

Bacteriocins represent a heterogeneous group of small, ribosomal-synthesized, potent antibacterial peptides produced by bacteria, capable of inhibiting bacteria both closely related or indeed those from other genera than the producer. In addition to their role as food preservatives, bacteriocins have potent activity against medically significant pathogens and are considered attractive alternatives to antibiotics, due to their inherent heat stability, potency at nanomolar scale, resistance to proteases and low levels of acquired resistance in commercial applications. The aim of this thesis was to identify, purify and bioengineer novel bacteriocins from *Staphylococcus* and Lactic Acid Bacteria (LAB), with a view to expanding the number of bacteriocins currently available and exploring potential food and clinical applications.

Firstly, a literature review examines the current and potential application of Staphylococcal derived bacteriocins in food preservation, medical and veterinary fields. This review introduces and describes some of the Staphylococcal derived bacteriocins, including their genetic organisation, mode of action and peptide structures. A second literature review examines the issues associated with nisin resistance, and the ability of pathogens to develop resistance to nisin that poses complications in relation to future applications. The first research chapter (Chapter 2) used traditional antimicrobial compound screening methods and the newer *in silico* genome mining tools to facilitate identification of a novel antimicrobial candidates in the genus *Staphylococcus*. This resulted in the discovery of the novel Class II bacteriocin capidermicin. The second research chapter (Chapter 3) sought to investigate the full potential of capidermicin to inhibit *Listeria Monocytogenes*. This ultimately resulted in the discovery of a remarkable "peptide partnership" with nisin which significantly reduced *Listeria monocytogenes* cell

numbers in food models. The third and final research chapter (Chapter 4) sought to bioengineer nisin to create new enhanced peptides. This resulted in the creation of nisin derivatives with dual functionalities that can overcome bacterial resistance mechanisms, in addition to enhanced antimicrobial activity against pathogenic bacteria.

This thesis seeks to drive and generate interest in bacteriocin discovery and application in both academia and industry. Furthermore, the studies contained provide direction for future development within this field and demonstrate the efficacy of bacteriocin use in food and clinical applications.

Chapter 1. Literature Review

The potential of Staphylococcal derived antimicrobials: in food preservation, medical and veterinary applications

1.1 Introduction

The rapid emergence and dissemination of resistant bacterial species continue to occur globally, threatening the longevity of antibiotics in the medical industry. In particular, problematic nosocomial infections caused by multi-drug resistant pathogens present as a major burden to both patients and healthcare systems, with annual mortality rates rising (Meade et al., 2020). Alarmingly, reports show an alarming trend where antimicrobial resistant infections result in 700,000 fatalities yearly, with 10 million deaths predicted by 2050 if this trend continues (Nicolaou and Rigol, 2018). Certainly, in order to combat the issue of antimicrobial resistance and multi-drug resistance bacteria, novel approaches must be sought out to curb infectious disease, control pathogenic species and ensure public health safety. Bacteriocins have received much attention in this regard.

By 2050, the global population is projected to reach 9.6 billion (Bradshaw and Brook, 2014) and this will bring about increased demands for food. Ensuring a safe and secure global food supply represents a major challenge to food manufacturers. In order to meet these demands, the global food supply may need to grow by as much as 70% (United Nations, 2013). Additionally, the global crop supply may have to increase by as much as 110% (Ray et al., 2013). To satisfy these increased demands, foodborne contamination must be diminished. Foodborne disease continues to remain a significant burden to food manufacturers. The infectious disease surveillance systems of the Centres for Disease Control and Prevention (CDC) estimates that each year approximately 10 million cases of foodborne illness occur in the United States as a result of microbially contaminated foods (Scallan et al., 2011). In 2015, 26 European countries reported a total of 4,362 foodborne outbreaks. Overall, these outbreaks were responsible for 45,874 illnesses, 3,892 hospitalisations and 17 deaths (EFSA, 2016).

In addition to public health burdens, the costs associated with foodborne disease, including hospitalization and treatment costs and product recalls, pose a significant financial impact. According to the United States Department of Agriculture (USDA), the annual costs associated with food-borne illnesses is between $10-83 billion dollars (Nyachuba, 2010). For Australia and New Zealand, the annual cost of foodborne illnesses has been estimated to be $1.289 billion and $86 million dollars, respectively (Lake et al., 2010; McPherson et al., 2011). In Europe, the yearly cost of foodborne illness was estimated to be $171 million USD in Sweden (Toljander et al., 2012), $2 million USD in Croatia (Razem and B Katusin-Razem, 1994) and £1.5 billion GBP for both England and Wales (O'Brien, 2014).

In order to prolong the shelf-life of foods, food preservatives are added to inhibit bacterial growth and prevent contamination. Most food preservatives are manufactured through chemical synthesis, and their long-term consumption can negatively impact the gut microbiota as they reduce bacterial counts (Sharma, 2015). Modern society has become more aware of the importance of food safety, as many of the chemical preservatives used in food may prompt side effects which can variety from mild to life threatening (Sharma, 2015). For example, the consumption of nitrites, which inhibit food spoilage bacteria in cured meats, has been identified as potentially causing stomach cancer (Song et al., 2015). The health benefits of natural foods without chemical preservatives has become increasingly popular among consumers, with many favouring minimally processed foods, and a 'preservative-free diet' (Sharma, 2015). However, such demands inherently lead to the increased risk of bacterial contaminated food products and foodborne illnesses. Unlike chemical additives, "generally recognized as safe" (GRAS) bacteriocins, including nisin, have been shown to be safe for use as food preservatives in

numerous food products, including vegetables, dairy, cheese, meats, and other food products, as they inhibit microorganism contamination during the food manufacturing process.

1.2 Bacteriocins: Our new "Silver Bullet?"

Bacteriocins represent a group of potent antimicrobial peptides that are ribosomally synthesized, many of which undergo post-translational modification. Bacteriocins are produced by bacteria to inhibit the growth of other bacteria, thus their production can provide an ecological advantage to the producing strain (Dobson et al., 2012). Bacteriocins are estimated to be produced by almost all bacteria (Riley and Wertz, 2002), with over 230 bacteriocins having been isolated and reported from the lactic acid bacteria alone, though an estimated 50% have been identified to the amino acid or DNA levels (Alvarez-Sieiro et al., 2016). BACTIBASE, an online web-based bacteriocin database, has 177 sequenced bacteriocins, including 156 from Gram-positive organisms and 18 from Gram-negative bacteria (Hammami et al., 2010) (http://bactibase.hammamilab.org/main.php). Antimicrobial peptides can be arranged into three groups based on their unique characteristics: Class I (modified), Class II (unmodified or cyclic) and Class III (>10 kDa peptides) (Cotter et al., 2005). Apart from their potent antimicrobial activity (often active in the micro- and nano-molar range), bacteriocins typically exert their antimicrobial activity against a narrow spectrum of bacteria. However, broad spectrum bacteriocins do also exist (e.g. Pep5, epidermin, epidermicin NI01 and aureocin A53 inhibit a range of Gram-positive pathogens) and these peptides may be suitable for treating bacterial infections with an unestablished causation. Alternatively, bacteriocins with a narrow spectrum of activity may allow for more precise targeting. Bacteriocins are gene-encoded, which make them highly

susceptible to bioengineering strategies. Compared to non-ribosomally synthesized peptides, the ribosomally produced peptides have many appealing properties, as they can be genetically manipulated to improve desirable characteristics such as potency, stability, solubility etc. (Field et al., 2019, 2010a, 2008; Rouse et al., 2012). Furthermore, they display low cytotoxicity and can be delivered as purified peptide or produced *in situ* by bacteriocin-producing bacteria (Cotter et al., 2013). Bacteriocins have been shown to interact with several bacterial surface receptors, which differ from antibiotic targeted receptors, reducing the risk of resistance development (Cotter, 2014).

1.3 The Staphylococcus genus

Staphylococcus species are Gram-positive cocci that are widely distributed throughout nature. Although they are frequently isolated from the skin and mucous membranes humans and animals (Bastos et al., 2009; Janek et al., 2016; Lauková et al., 2020). In general, *Staphylococcus* members have a symbiotic relationship with their host (Christensen and Bruggemann, 2014; Teruaki Nakatsuji et al., 2017). However, if they gain entry into the host tissue, they develop the lifestyle of an opportunistic pathogen. Numerous *Staphylococcus* species including *S. aureus, S. epidermidis, S. lugdunensis and S. saprophyticus* are frequently identified as causative agents of numerous human and animal infections (Otto et al., 2010). Staphylococci strains resistant to the antibiotic methicillin have become important nosocomial pathogens, and require more effective measures for containment.

Staphylococci can be arranged into two separate groups, coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS) based on their

ability to coagulate blood through production of coagulase (Bannerman and Peacock, 2007), a characteristic which is associated with pathogenicity.

The *Staphylococcus* family consists of 52 known species with 28 subspecies, for which CoNS are the most prevalent. So far, there are eight species known to be coagulase-positive, including *S. aureus, S. delphini*, *S. hyicus* subsp. *hyicus* and *chromogenes, S. intermedius, S. pseudintermedius, S. lutrae* and *S. leei* (Schleifer and Bell, 2015). CoNS are members of the commensal bacteria of the skin and thought to provide the host with protection against invading pathogenic bacteria (Christensen and Bruggemann, 2014). Nakatsuji and colleagues (T Nakatsuji et al., 2017) revealed that antimicrobial – producing CoNS were absent in atopic dermatitis patients, and re-establishment of these producing strains decreased the colonization of pathogenic *Staphylococcus aureus*.

1.4 Staphylococcal bacteriocin Classification

Staphylococcal species can produce antimicrobial peptides which are commonly referred to as staphylococcins. The term ‘staphylococcin’ originated in 1946, after the description of antimicrobial compounds produced by staphylococci inhibited the growth of Gram-positive bacteria (Fredericq, 1946). Overall, the structure, mode of action and the genetic elements of staphylococcal-derived antimicrobials have been investigated less than other bacteriocins, especially when compared to Lactic Acid Bacteria (LAB). The majority of staphylococcins thus far studied are produced by both CoNS and CoPS species and belong to class I and class II bacteriocins: Class I, lantibiotics (sixteen members, all from CoNS strains), Class II (eight staphylococcins), Class III (comprising two bacteriocins) and Class IV (one bacteriocin - aureocyclicin 4185) (see Table 1).

Although various reviews have been published on bacteriocins produced by Gram-positive bacteria, they commonly refer to aspects related to those antimicrobial peptides produced by lactic acid bacteria (LAB), which are of eminent economic importance because of their widespread use within the food sector. A recent review by Newstead and colleagues (Newstead et al., 2020), discusses the family of staphylococcal-derived antimicrobial peptides, and highlights their potential as alternative treatments for *S. aureus* infections. Therefore, this present review will seek to provide an overview of the diversity of bacteriocins produced by *Staphylococcus* species and their potential for use in food preservation, clinical and veterinary applications.

Table 1: Relevant characteristics of Staphylococcal bacteriocins.

Bacteriocin	Length	Mw (Da)[1]	pI[2]	Net charge[2]	Producing strain	Reference
Class I – lantibiotic peptides						
Pep5	34	3488	11.08	+8	*S. epidermidis* strain 5	(Bierbaum, 1996)
Epidermin	22	2164.6	8.33	+2	*S. epidermidis* Tü3289	(Allgaier et al., 1986)
Gallidermin	21	2165	8.33	+2	*S. gallinarium* DSM4616	(Schnell, 1989)
Staphylococcin C55 Alpha	30	3336	7.12	+1	*S. aureus* C55	(Navaratna et al., 1998)
Staphylococcin C55 Beta	32	2993	8.04	+1		
Epicidin 280	30	3130	10.35	+5	*S. epidermidis* BN280	(Heidrich et al., 1998)
Epilancin K7	31	3032	10.30	+6	*S. epidermidis* K7	(Kamp et al., 1995)
Epilancin 15X	31	3170	10.80	+8	*S. epidermidis* 15X154	(Ekkelenkamp et al., 2005)
Nukacin ISK-1	27	2960	8.53	+4	*S. warneri* ISK-1	(Sashihara et al., 2000)
Nukacin 3299	27	2960	8.53	+4	*S. simulans* 3299	(Ceotto et al., 2010)
Nukacin IVK45	27	2940	8.35	+2	*S. epidermidis* IVK45	(Janek et al., 2016)
Hyicin 3682	22	2139	7.75	+1	*S. hyicus* 3682	(Fagundes et al., 2011)
Nisin J	35	3458	8.67	+4	*S. capitis* APC2923	(O'Sullivan et al., 2020)
BacCh91	22	2075	7.92	+1	*S. aureus* CH9/DSM26258	(Wladyka et al., 2013)
Hominicin	21	2038	3.85	+1	*S. hominis* MBBL2–9	(Kim et al., 2010)
Warnericin RB4	27	2958	N/A	N/A	*S. warneri* RB4	(Minamikawa et al., 2005)
Hyicin 4244	35	3274	4.03	-2	*S. hyicus* 4244	(Duarte et al., 2018)
Single peptide class II leaderless bacteriocins						
Aureocin A53	51	6012.19	10.73	+8	*S. aureus* A53	(Netz et al., 2002a)

Bacteriocin	Length	Mw (Da)[1]	pI[2]	Net charge[2]	Producing strain	Reference
Epidermicin NI01	51	6072.27	10.95	+9	*S. epidermidis* strain 224	(Sandiford and Upton, 2012)
BacSp222	50	5921.92	10.09	+4	*S. pseudintermedius* strain 222	(Wladyka et al., 2015)
Capidermicin	50	5466	10.22	+5.34	*S. capitis* CIT060	(Lynch et al., 2019)
Multi-peptide Class II leaderless bacteriocins						
Aureocin A70					*S. aureus* A70	(Netz et al., 2001)
Aur70A	31	2952.53	10.98	+4		
Aur70B	30	2825.34	10.87	+4		
Aur70C	31	2982.56	10.87	+5		
Aur70D	31	3114.76	10.70	+4		
Class III Staphylolytic bacteriocins						
Lysostaphin	246	26,926	9.5		*S. simulans* biovar *staphylolyticus*	(Schindler and Schuhardt, 1964)
ALE-1	362	35,596	9.61		*S. capitis* EPK-1	(Sugai et al., 1997a)
Class IV bacteriocins						
Aureocyclicin 4185	60	5607	10	+2	*S. aureus* 4185	(Potter et al., 2014)

[1] The calculated molecular weight of peptides includes the N-formylated methionine, where applicable.

[2] Information relating to molecular weight, pI and charge using Bactibase (http://bactibase.hammamilab.org/main.php) and GeneScript® Peptide Property Tool.

1.5 Class I lantibiotic peptides produced by *Staphylococcus*

1.5.1 Pep5

Pep5 is a lantibiotic peptide produced by *S. epidermidis* strain 5 (Bierbaum et al., 1994). Pep5 is cationic, composed of 34-amino-acids residues and has a confirmed molecular weight of 3,488 Da (Bierbaum et al., 1994). Kellner and colleagues (Kellner et al., 1989) determined the structure of Pep5 and determined that the peptide consists of three ring structures (one MeLan and two Lan), two dehydrobutyrine (Dhb) residues in the central area of the peptide, and an oxobutyryl residue located at the N-terminus (Fontana et al., 2006; Kellner et al., 1989). Pep5 has been shown to prevent the adherence and colonisation of clinical *S. epidermidis* and *S. aureus* to medical devices, including catheters (Fontana et al., 2006). The eradication of staphylococcal isolates from the surface of medical devices demonstrates the promising potential for Pep5 to be utilised within to the medical device sector, thus avoiding the nosocomial bloodstream infections.

1.5.2 Epicidin 280

Epicidin 280 is a peptide produced by *Staphylococcus epidermidis* BN280 (Heidrich et al., 1998). Epicidin 280 consists of 30 amino acid residues and has a confirmed molecular weight of 3,130 Da (Heidrich et al., 1998). Epicidin 280 displays 75% sequence similarity to the lantibiotic Pep5, and thus is considered a natural variant of Pep5 (Heidrich et al., 1998). Epicidin 280 displays strong antimicrobial activity towards numerous nosocomial CoNS strains associated with human infection, including *S. epidermidis*, *S. hominis*, *S. saprophyticus*, *S. sciuri* and *S. simulans* (Nascimento et al., 2006).

1.5.3 Epilancin K7 and 15X

Epilancin K7 is a lantibiotic peptide produced by *S. epidermidis* K7, consists of 31 amino acids and has a determined molecular weight of 3,032 Da (Van De Kamp et al., 1995). Epilancin 15X is lantibiotic produced by *S. epidermidis* 15X154, that was isolated from a bacterial infection (Ekkelenkamp et al., 2005). Epilancin 15X consists of 31 amino acid residues (Figure. 1), and has a confirmed molecular weight of 3,170 Da (Ekkelenkamp et al., 2005). Epilancin 15X displayed 68% sequence identity to epilancin K7, with three nearly identical lanthionine rings, similar distribution of positive charges, and contain a hydroxy-propiony at the N-terminus (Ekkelenkamp et al., 2005; Van De Kamp et al., 1995). The primary structure of epilancin 15X has been described as 'simplistic' when compared to other lantibiotic peptides, however epilancin 15X displays strong bioactivity against target bacteria. The epilancin 15X molecule contains an unusual D-lactate group located at the N-terminus (Figure 1), and it is thought to be important for antimicrobial activity. Additionally, it has been suggested that this unusual modification provides the peptide with proteolytic resistance against aminopeptidases (Velásquez et al., 2011).

Epilancin 15X has potent antimicrobial activity against numerous human pathogens, including methicillin-resistant *S. aureus* (MRSA), *S. epidermidis* and vancomycin-resistant Enterococci (VRE) (Velásquez et al., 2011). A study by Knerr and van der Donk (Knerr and Van Der Donk, 2012), used chemically synthesised epilancin 15X derivatives, in order to investigate the importance of several post-translational modifications at the N-terminus for antimicrobial activity. Unexpectedly, the alternation of the N-terminus with different moieties, including the d-lactyl group, had very little

effect on pore formation or bioactivity of the epilancin 15X peptide derivatives (Knerr and Van Der Donk, 2012).

1.5.4 Epidermin and its natural variants

Epidermin, along with its naturally occurring variants are some of the most commonly isolated lantibiotic peptides in the group of CoNS, which are isolated from numerous *S. epidermidis* strains, and other CoNS strains (Bastos et al., 2009; Bierbaum, 1996; Carson et al., 2017; Kumar et al., 2017). The epidermin is a lantibiotic peptide produced by *S. epidermidis* Tu3298, is composed of 22 amino acid residues, and contains four ring structures (Götz et al., 2014; Schnell et al., 1988). The antimicrobial activity was confirmed in the cell free supernatant, subsequently purified and a molecular weight of 2,164.6 Da was determined by MALDI-TOF MS analysis (Allgaier et al., 1986). Epidermin demonstrates strong bioactivity against several pathogenic bacteria, including *Staphylococcus*, *Streptococcus* and *Propionibacterium acnes* (Kellner et al., 1988), and clinical investigations have revealed the potential application of epidermin for treatment of acne (Bonelli et al., 2006). The potent antimicrobial activity of epidermin, often at nano-molar concentrations, can be attributed to its highly efficient dual mode of action, which involves interrupting cell wall biosynthesis (through binding Lipid II) and disrupting the cellular membrane (through pore formation) (Bonelli et al., 2006).

Gallidermin is a natural variant of epidermin, which is produced by *S. gallinarium* DSM4616 (Kellner et al., 1988). Gallidermin and epidermin are structural analogues which differ at position 6 in the N-terminal region of the peptides (see Figure 1) (Götz et al., 2014; Kellner et al., 1988). In a similar way to epidermin, gallidermin has a dual mode of action, involving interacting with the membrane, pore formation and the inhibition of

membrane synthesis. This mode of action contributes to the potency of the peptide, with gallidermin capable of inhibiting problematic CoNS strains at peptide concentrations as low as 0.0156 μg (Lauková et al., 2020). Moreover, gallidermin was subjected to mutagenetic analysis to determine the importance of the modification enzymes and identify gallidermin derivatives with enhanced bioactivity (Ottenwalder et al., 1995). This study demonstrates that bioengineering strategies can generate enhanced gallidermin derivatives with improved functionalities, including increased antimicrobial activity and proteolytic resistance. However, more importantly, this bioengineering provided valuable information relating to the structural elements required for production of both epidermin and gallidermin (Götz et al., 2014; Ottenwalder et al., 1995).

In the staphylococcal genome mining study by Carson and colleagues (Carson et al., 2017) twenty-nine lantibiotic gene clusters were uncovered in CoNS genomes. Two lantibiotic clusters came from two *S. epidermidis* isolates, containing an identical 52 amino acid precursor peptide, including the epidermin-conserved domain and shared 96% identity with epidermin (Carson et al., 2017). Another related study by Kumar and colleagues (Kumar et al., 2017), isolated *S. capitis* TE8 from skin surface of a healthy human foot, genome sequencing analysis revealed a gallidermin-type lantibiotic peptide with 63% identity to gallidermin and 59% identity to epidermin.

During the screening of 187 CoNS isolates, a lantibiotic was identified from the supernatant of two *S. epidermidis* strains, termed BN-V1 and BN-V30 (Israil et al., 1996). Mass spectrometry analysis subsequently established a molecular weight of 2,151 Da for both peptides, which differs from all previous characterised antimicrobial peptides, with epidermin and gallidermin having a molecular weight of 2,165 Da (Israil et al., 1996).

Using N-terminal sequencing, the two lantibiotic peptides were confirmed as natural variants of epidermin and incorporates the amino acid difference at position six (similar to gallidermin) but includes novel amino acid variation at position one (Israil et al., 1996).

Staphylococcin 1580 is a lantibiotic produced by *S. epidermidis* 1580 (Jetten and Vogels, 1973; Sahl, 1994). The bioactivity of staphylococcin 1580 was confirmed in the cell free supernatant, and subsequently purified, revealing a peptide identical to epidermin (Sahl, 1994). Staphylococcin T was isolated from *S. cohnii* T , which is identical to gallidermin (Furmanek et al., 1999).

Taken together, these studies demonstrate that the production of epidermin and its natural derivatives appears to be widespread among CoNS strains.

1.5.5 Nukacin ISK-1 and nukacin-like bacteriocins

Nukacin ISK-1 is a lantibiotic peptide that is produced by *S. warneri* ISK-1, which was originally isolated from fermented rice bran (Fujinami et al., 2018; Kimura et al., 1998). Nukacin ISK-1 consists of a 57 amino acid precursor, and 27 amino acid bioactive peptide (Figure. 1) and has a confirmed molecular weight of 2,960 Da (Kimura et al., 1998). Nukacin ISK-1 contains a conserved region similar to the lipid II binding region of mersacidin, produced by *Bacillus* sp. strain HIL Y-85,54728 (Brotz et al., 1995), signifying a shared mode of action. Preliminary studies seem to indicate that nukacin ISK-1 displays a bacteriostatic mode of action caused by binding to lipid II which inhibits cell wall biosynthesis (Islam et al., 2012). This has been recently confirmed along with the illumination of other important residues in the peptide (Elsayed et al. 2017). Moreover, a site-saturation mutagenesis strategy was employed to reveal the importance of the amino acids of nukacin ISK-1(Islam et al., 2009). This bioengineering approach

identified two nukacin ISK-1 derivatives, termed D13E and V22I, which demonstrated two-fold greater specific bioactivity against a number of target strains, including *Lactobacillus sakei* ssp. *sakei* JCM 1157, *Bacillus coagulans* JCM 2257, *Pediococcus pentosaceus* JCM 5885, *Enterococcus faecalis* JCM 5803 (Islam et al., 2009).

Nukacin-like bacteriocins have also been identified in *S. simulans* (Carson et al., 2017; Ceotto et al., 2010b), *S. hominis* (Wilaipun et al., 2008), and *S. chromogenes* (Braem et al., 2014). Janek and colleagues (Janek et al., 2016) identified a nukacin-like lantibiotic from *S. epidermidis* IVK45. Nukacin IVK-45 consists of 27 amino acids, and has a confirmed molecular weight of 2,940 Da (Janek et al., 2016). Nukacin IVK-45 differs from nukacin ISK-1 by five amino acids in the mature peptide and another five amino acids in the leader sequence (Ala6Val, Val7Ile, Trp19Phe, Ile22Val, Gly27Ser) (Janek et al., 2016).

1.5.6 Staphylococcin C55

Navaratna and colleagues (Navaratna et al., 1998) identified the first two component staphylococcal-derived lantibiotic. Staphylococcin C55 is produced by *S. aureus* C55, and produces two distinct peptides, termed C55α and C55β (Navaratna et al., 1998). Both peptides, C55α and C55β were confirmed to be lantibiotics through N-terminal sequencing, with determined molecular masses of 3,339 Da and 2,993 Da, respectively (Navaratna et al., 1998). Interestingly, when both lantibiotic peptides are used in combination, C55α and C55β work synergistically to inhibit *S. aureus* and *Micrococcus luteus* (Navaratna et al., 1998).

1.5.7 Nisin J, a natural nisin variant

Nisin J is a lantibiotic nisin variant produced by *Staphylococcus capitis* APC2923, a strain isolated from the skin microbiota (O'Sullivan et al., 2020). The antimicrobial activity of nisin J was obtained from the cell free supernatant, subsequently purified using a combination of column chromatography and RP-HPLC, subjected to mass spectrometry analysis which confirmed a molecular weight of 3458 Da (O'Sullivan et al., 2020). Nisin J differs from nisin A by nine amino acid changes (Ile4Lys, Met17Gln, Gly18Thr, Asn20Phe, Met21Ala, Ile30Gly, Val33His and Lys34Thr) and contains an extra amino acid at the C-terminus (O'Sullivan et al., 2020). Nisin J displayed potent antimicrobial activity against MRSA, and was demonstrated to more effective at inhibiting indicators strains than nisin A (O'Sullivan et al., 2020). However, no difference was observed when nisin J was compared with other natural nisin variants, including nisin A and Z, using broth-based MIC determinants against *L. lactis* HP (O'Sullivan et al., 2020).

1.6 Staphylococcal lantibiotics - Genetics, structural features and biosynthesis

The Class I lantibiotics represent the most abundant group of staphylococcal-derived antimicrobials peptides. Lantibiotics, or lanthionine containing antibiotics, are characterised by the occurrence of uncommon amino acids, including lanthionine (Lan) and β-methyllanthionine (MeLan) (Cotter et al., 2013). These unusual amino acid residues are formed post-translationally through enzymatic dehydration of serine or threonine to form didehydroalanine (Dha) or didehydrobutyrine (Dhb), respectively, which then react, via their double bonds, with the thiol groups of neighbouring cysteine residues to form the non-standard amino acids lanthionines and β-methyl-lanthionines. These unusual residues form covalent bridges which results in internal rings, which

provides lantibiotic peptides with their distinguishing structural composition (see Figure 1).

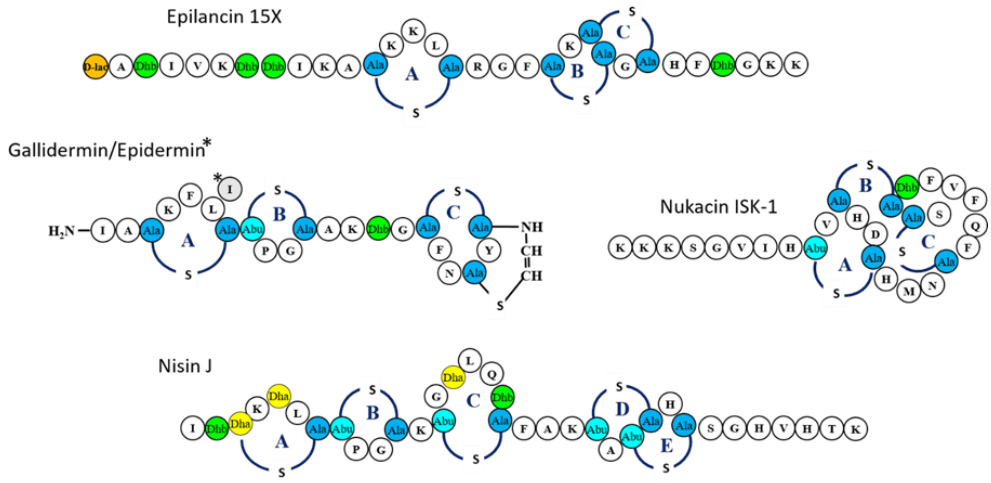

Figure 1. The primary structures of Epilancin 15X, epidermicin/gallidermin, nukacin ISK-1 and Nisin J. Coloured circles indicate amino acid residues: Abu, α-amino butyric acid; Dhb, dehydrobutyrine; Abu-S-A, methyllanthionine, and A-S-A, lanthionine, where 'S' denotes a mono-sulphide linkage.

*Gallidermin differs from epidermin at position 6 (Ile).

All the genes essential for the production, transport, and secretion of a lantibiotic peptide can be found within the gene cluster (operon). Lantibiotic production involves numerous stages, including translation of the prepeptide, dehydration and cross-linkage of amino acid residues, cleavage of the leader sequence, and secretion of the bioactive peptide (McAuliffe et al., 2001). Initially, lantibiotics are synthesized as inactive prepeptides, which incorporate an N-terminal leader sequence attached to the C-terminal pro-peptide. During the maturation process, the leader sequence is removed and the C-terminal propeptide undergoes post-translational modifications to produce a bioactive

lantibiotic. It has been proposed that the leader sequence has many roles, including providing a recognition motif for the post-translational modifications, assisting in folding the prepeptide, protection against degradation, and maintaining an inactive peptide until the appropriate time for transport and secretion of a bioactive lantibiotic (Arnison et al., 2013; Oman and Van Der Donk, 2010). Notably, some two-component lantibiotics require the two peptides to work synergistically in order to achieve antimicrobial activity. These peptides undergo post-translational modification by separate modification enzymes to achieve a synergistic activity outside of the cell (Bierbaum and Sahl, 2009). For certain lantibiotics, including epidermin and Pep5, post-translational modifications are orchestrated by LanB and LanC. Firstly, the conversion of serine and threonine to dehydroalanine (Dha) and dehydrobutyrine (Dhb) respectively, is managed by LanB (McAuliffe et al., 2001). LanC then generates a thioether bond formation involving thiol groups of nearby cysteine residues and the double bonds of Dha and Dhb to form lanthionine and methyllanthionine, respectively (see Figure 2). However, in the case of some lantibiotics these specific modification reactions using a single modification enzyme encoded by *lanM* (Arnison et al., 2013; Begley et al., 2009; Carson et al., 2017). Subsequently, the leader sequence of the modified pre-lantibiotics are proteolytically removed, leading to a fully biologically active mature lantibiotic peptide (McAuliffe et al., 2001). For lantibiotic peptides modified by LanB and LanC, this is achieved by a dedicated serine protease LanP which can be situated intracellularly or extracellularly, e.g. for Pep5 the PepP protease is located intracellularly (Meyer et al., 1995) and proteolytic processing occurs within the cell prior to export whereas EpiP (epidermin) are extracellular proteases and activate the lantibiotics following export (Figure 2). The LanT proteins belong to the ABC-superfamily of transport proteins, and are mainly involved in

the transport of the lantibiotic outside the producing cell to surrounding environment where they will be bioactive peptides (see Figure 2). Specific immunity proteins are essential for protecting the producing strain against the bioactivity of its own peptide. Lantibiotic self-protection mechanisms are governed by LanI, LanH and LanFEG (Götz et al., 2014). For some lantibiotics, including nisin, lantibiotic production is controlled through a two-component regulatory system consisting of *lan*K and *lan*R genes, which encode a histidine protein kinase (HK) and a cytoplasmic response regulator (RR), respectively (Bierbaum et al., 1996; Ebner et al., 2018; Götz et al., 2014). The HK and RR proteins are both involved in lantibiotic regulation by a quorum sensing system (Champak Chatterjee et al., 2005). Presence of active lantibiotic peptide leads to a signalling cascade initiated by LanK, and subsequent activation of LanR to stimulate biosynthesis of the bacteriocin (Dischinger et al., 2014). However, it's worth noting, that not all these biosynthetic genes are found in all lantibiotic gene clusters. Notably, the N-terminus of epilancin 15X contains an unusual d-lactyl (DLac) cap, which is applied by the oxidoreductase ElxO via stereospecific reduction of a pyruvyl moiety generated from hydrolysis of an N-terminal Dha (Velásquez et al., 2011).

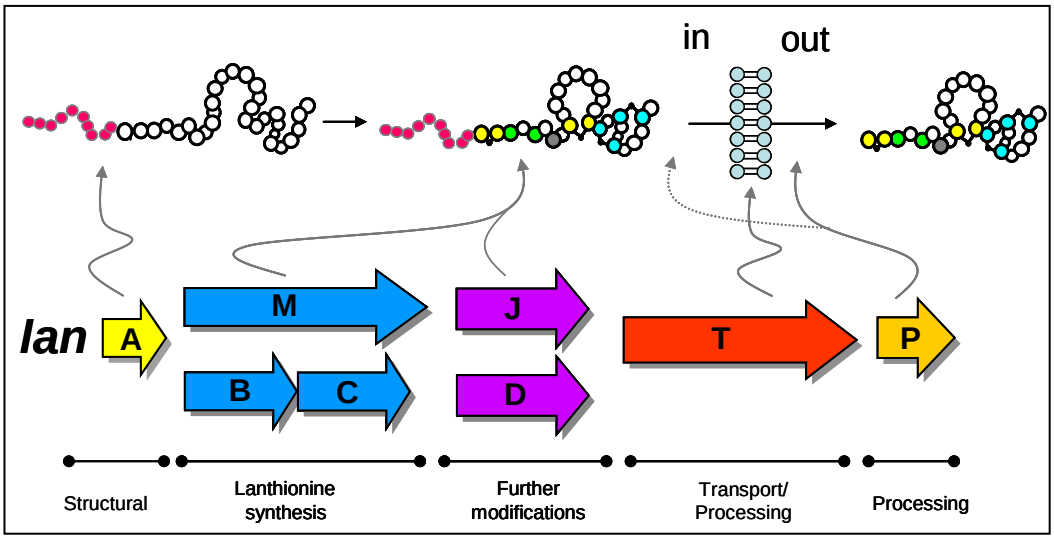

Figure 2. Lantibiotic biosynthesis taken from Field et al, 2010. The prepeptide, LanA, is produced from the structural gene. For lantibiotics, the LanB and LanC enzymes induce the dehydration of serine and threonine residues and Lan/MeLan formation, respectively. For other lantibiotics, LanM, performs modification reactions. Further modifications may also occur, LanJ allows for the formation of d -alanines and the C-terminal cysteine is decarboxylated and converted into an S-amino vinyl-D-cysteine by the LanD enzyme. Following modifications, lantibiotic peptides are transported out of the cell using LanT, and the leader sequence is removed by LanP. For some lantibiotics, LanT performs both roles.

The biosynthetic gene clusters of gallidermin and epidermin are quite similar, both comprise eleven genes that are localized on a large plasmid (Götz et al., 2014). However, they are distinguished by the presence of the unusual genes *epiH* and *gdmH*, which encode for membrane proteins that enable the secretion of the bioactive peptides (Götz et al., 2014; Peschel and Götz, 1996). The ABC transport system, typically involved in the transport of lantibiotics, is absent in the epicidin 280 gene cluster (Heidrich et al., 1998).

Although the gene responsible for the transport of epicidin 280 is not found within the epicidin 280 lantibiotic operon, it is presumed that a host transport system takes over this function (Bierbaum et al., 1994; Meyer et al., 1995).

The epilancin K7 biosynthetic gene cluster resembles the genetic organization of Pep5 (Van De Kamp et al., 1995). However, in the pep5 gene cluster, a smaller gene is located between the *pepA* and *pepT* encoding for an immunity protein (*pep*I) (Heidrich et al., 1998). However, no gene which would be comparable to *pep*I, has been identified between the *elkA* and *elkT* genes (Bastos et al., 2009; Van De Kamp et al., 1995). The biosynthetic operon of epilancin 15X resembles that of the Pep5 (Meyer et al., 1995) and epicidin 280 (Heidrich et al., 1998), suggesting that these lantibiotics may have evolved from a common ancestor.

Nukacin ISK-1 is a member of the lacticin 481 group of lantibiotics and in contrast to other lantibiotics, the modification enzyme LanM carries out the dehydration and cyclization of the peptide (Aso et al., 2004). The multi-functional ABC transporter NukT executes the removal of the leader sequence and transport of the peptide, which produces the bioactive peptide (Aso et al., 2004). The expression of nukFEG or nukH alone provided only partial self-immunity, however when both *nuk*FEG and *nuk*H are expressed a significantly higher degree of self-immunity is generated (Aso et al., 2005). These findings suggest that *nuk*H works synergistically with *Nuk*FEG to provide self-immunity. Interestingly, the nukacin ISK-1 self-immunity system provided immunity against lacticin 481, but not against nisin (Aso et al., 2005).

Analysis of the nisin J operon revealed several key features associated with lantibiotic operons (O'Sullivan et al., 2020). However, nisin J differs from other natural

nisin variants, in that it lacks the lantibiotic regulatory and immunity genes, *nis*RK and *nis*I, and order of genes within the operon (O'Sullivan et al., 2020).

1.7 Mode of action of Staphylococcal lantibiotics

The ability of cationic peptides to disrupt membranes with a negative surface charge has been well documented (Netz et al., 2002b). Although the mode of action of some bacteriocins is still under investigation it is generally accepted that many class I and class II bacteriocins function like other cationic amphipathic peptides by interacting with anionic membrane phospholipids, causing pore formation and leading to interruption of the cell membrane, cell metabolite leakage causing cell death (Bastos et al., 2009; Paul D Cotter et al., 2005). Lantibiotic peptides, like nisin, which are linear positively charged peptides, have dual modes of action where they inhibit cell wall biosynthesis and form pores that mainly act upon Gram-positive bacteria (Bierbaum and Sahl, 2009). In relation to nisin, which has been studied extensively, the cell wall precursor lipid II is the target and upon binding, nisin inhibits peptidoglycan synthesis. Other staphylococcal lantibiotic peptides such as epidermin and gallidermin also bind to lipid II, but are too short to span the lipid bilayer to form pores, yet are still able to kill target bacteria, indicating that they have other lipid II mediated mechanisms (Bastos et al., 2015). Gallidermin displays a unique mode of action, in that it causes a lipid 'flip flop', i.e. gallidermin causes lipid II to 'flip' from the inside to the outside of the membrane, and thus, cannot be incorporated into the peptidoglycan layer (Götz et al., 2014). For pep5 and epilancin K7, specific receptor molecules and/or surface targets have yet to be identified (Draper et al., 2015).

1.8 Class II bacteriocins produced by *Staphylococcus*

1.8.1 Leaderless bacteriocins

Leaderless bacteriocins represent a particularly unusual group of antimicrobial peptides. Generally, bacteriocins are synthesized as precursor peptides containing an N-terminal leader sequence, however, as the name might suggest, leaderless bacteriocins are synthesized without a leader sequence. Nevertheless, leaderless bacteriocins are ribosomally-produced and possess distinctive features which allows them to have homology with bacteriocins from other bacteriocin classes (Perez et al., 2018). Moreover, their simplicity has generated interest for the easy expression potential in any expression system, and scale-up production systems. Interestingly, one shared characteristic among the majority leaderless bacteriocins is the presence of an N-formylated methionine (Perez et al., 2018). However, the significance of this characteristic in the biosynthesis and/or antimicrobial remains unclear. In 2016, Acedo and colleagues (Acedo et al., 2016) demonstrated the retention of antimicrobial activity following the removal of the N-formyl-methionine, revealing its unimportance with respect to bioactivity of the peptide.

In 1998, Cintas and colleagues (Cintas et al., 1998) reported the first leaderless bacteriocin. Enterocin L50, produced by *Enterococcus faecium* L50, consists of two peptides that work in a synergistic manner (Cintas et al., 1998). However, these peptides are synthesized without a leader sequence, and this distinct feature, distinguishes them from other two-peptide bacteriocins. In recent years, an ever-increasing number of studies have described the isolation and characterisation of leaderless bacteriocins (Iwatani et al., 2007a; Kumar et al., 2017; Lozo et al., 2017; Netz et al., 2002c; Sandiford and Upton, 2012), thus proposing that leaderless bacteriocins could represent a large group of antimicrobials. Many leaderless bacteriocins have already been reported that are single

peptide, two-peptide and multi-peptide. Members of this Class II group of antimicrobials include aureocin A53, BHT-B, enterocin Q, epidermicin NI01, lacticins Q and Z, lactolisterin BU, LsbB, weissellicins Y and M (Fujita et al., 2007; Gajic et al., 2003; Hyink et al., 2005; Iwatani et al., 2007a; Lozo et al., 2017; Masuda et al., 2012; Netz et al., 2002a; Sandiford and Upton, 2012).

1.9 Class II bacteriocins produced by *Staphylococcus*

1.9.1 Aureocin-like peptides

Aureocins are an emerging group of antimicrobial peptides with a broad spectrum of activity and were first isolated from *Staphylococcus aureus*. Aureocins act bactericidally on target bacterial cells, causing rapid lysis (Fagundes et al., 2016; Netz et al., 2002a; Perez et al., 2018). Based on their structure, they can be categorised into two main groups: single-peptide aureocins (aureocin A53-likepeptides), or multi-peptide aureocins (aureocin 70-likepeptides).

1.9.2 Aureocin A53

Aureocin A53 is single-peptide leaderless bacteriocin produced by *S. aureus* A53, which was originally isolated from a milk sample (Giambiagi-Marval et al., 1990; Netz et al., 2002a). Aureocin A53 is a cationic, tryptophan-rich peptide (containing ten lysine and five tryptophan residues) and has a net charge of +8 (Netz et al., 2002a). The antimicrobial activity was confirmed from the bacterial cell-free supernatant, subsequently purified, and mass spectrometry determined a molecular weight of 6,021.5 Da, which was 28 Da higher than predicted (Netz et al., 2002c). The mass increase can be accounted for by the presence of a formylated methionine residue at the N-terminus, indicating that aureocin A53 is synthesized and transported without a leader sequence

(Netz et al., 2002c). In addition to potent bioactivity and a broad activity spectrum, aureocin A53 displays impressive protease stability (Netz et al., 2002a, 2002c).

Aureocin A53 has been shown to carry out its antimicrobial activity through infiltrating bacterial membranes, however not through pore formation (Netz et al., 2002a). Notably, the cationic peptide displayed a stronger affinity for the neutral bacterial membrane than the negatively charged phospholipids (Perez et al., 2018). Initially, it was postulated that the potent bioactivity of aureocin A53 was attributed to the electrostatic interaction of the cationic peptide and the phospholipid membrane, however this finding has removed that theory. Nonetheless, this Staphylococcal leaderless bacteriocin has a strong bioactivity against many pathogenic bacteria, including *Enterococcus*, *Listeria* and *Staphylococcus* (Netz et al., 2002a; Perez et al., 2018).

1.9.3 Epidermicin NI01

Sandiford and Upton (Sandiford and Upton, 2012) isolated and characterised a leaderless bacteriocin from a human isolate *S. epidermidis* strain 224, called epidermicin NI01. Epidermicin NI01 is highly cationic, hydrophobic peptide consisting of four tryptophan and nine lysine residues (Sandiford and Upton, 2012). The peptide is composed of 51 amino acids, and displays 50% sequence identity to other Class II bacteriocins, including aureocin A53, and lacticin Q and Z (Sandiford and Upton, 2012). Epidermicin NI01 displays potent bioactivity against a range of problematic pathogenic bacteria including methicillin-resistant *S. aureus* (MRSA), *enterococci*, and *S. epidermidis*, with MIC values ranging from 0.0625 – 4 µg/ml (Sandiford and Upton, 2012). In 2017, it was revealed that a single application of epidermicin NI01 was sufficient to control and prevent the colonisation of MRSA in the nares of rats (Halliwell et al., 2017).

1.9.4 BacSp222

A study conducted by Wladyka and colleagues (Wladyka et al., 2015) describe a unique peptide, BacSp222, is produced by *S. pseudintermedius* strain 222 isolated from dog skin lesions. This cationic peptide is composed of fifty amino acid residues, rich in tryptophan residues and shares sequence similarities to other class II peptides such as aureocin A53, lacticins Q and Z, epidermicin NI01 (Wladyka et al., 2015). BacSp222 display potent anti-staphylococcal activity at micro-molar concentrations. However, higher concentrations of BacSp222 are cytotoxic towards eukaryotic cells (Wladyka et al., 2015). The antimicrobial activity was obtained from the cell free supernatant, subsequently purified using a combined of ammonium sulphate and RP-HPLC and MALDI-TOF MS analysis confirmed a molecular mass of 5921.92 Da (Wladyka et al., 2015). The purification strategy employed produced 0.7 mg of purified peptide from 1 litre of cell free supernatant (Wladyka et al., 2015). Additionally, similar to aureocin A53, differences in the predicted molecular weight were accounted for by the presence of a N-terminal formylated methionine.

1.9.5 Capidermicin

Traditional agar based screening methods were utilized by Lynch and colleagues (Lynch et al., 2019) in the isolation of capidermicin. Capidermicin is a cationic peptide, produced by *S. capitis* CIT060, and is composed of fifty amino-acid residues (Lynch et al., 2019). Capidermicin is plasmid-encoded and exhibits sequence similarities to other characterised bacteriocins including lacticin Z (46% identity), aureocin A53 (41% identity) and an aureocin-like bacteriocin (57% identity) (Lynch et al., 2019). Initially, bioactivity was confirmed in the cell free supernatant, subsequently capidermicin was purified using a combination of column chromatography and RP-HPLC, and a molecular

weight of 5,466 Da was determined by MALDI-TOF MS analysis (Lynch et al., 2019). Capidermicin exhibits potent bioactivity against a range of Gram-positive bacteria, with MIC values ranging from 0.6 - 18µM (Lynch et al., 2019). Subsequently, the isolation, characterisation and potential applications of this novel peptide will be described in this thesis (Chapter 2).

1.10 Multi-peptide aureocin-like peptides

1.10.1 Aureocin A70

Aureocin A70 is a bacteriocin produced by *S. aureus* A70, originally isolated from milk (Netz et al., 2001). Aureocin A70 consists of four class II leaderless peptides with similar molecular weights and amino acid length, including Aur70A (31 amino acids), Aur70B (30 amino acids), Aur70C (31 amino acids) and Aur70D (31 amino acids) (see Table 1) (Coelho et al., 2014; Perez et al., 2018). The antimicrobial potential of each chemically synthesized peptide was assessed using deferred antagonism assays (Bastos et al., 2009). When examined individually against *Listeria innocua* and *S. aureus* strains, no antimicrobial activity (zones of inhibition) was reported, however a significant improvement in bioactivity (larger inhibition zones) was revealed when all four peptides were combined. Further antimicrobial investigations with *Micrococcus luteus* revealed that Aur70A, Aur70B, and Aur70C displayed bioactivity individually (60 µg), with a decreasing effectiveness as follows, Aur70A > Aur70B > Aur70C, with Aur70D exhibiting no antimicrobial activity (Bastos et al., 2009). However, the combination of all four peptides caused a greater increase in antimicrobial activity, i.e. bigger inhibition zones (Bastos et al., 2009), indicating aureocin A70 requires all four peptides to work synergistically for inhibition to occur.

1.11 Genetic organisation and biosynthesis of leaderless bacteriocins

To date, there is a lack of knowledge on the biosynthesis of leaderless bacteriocins, despite several publications outlining the genetic organisation of a number of leaderless bacteriocins. The genes responsible for bacteriocin immunity and secretion have been experimentally confirmed for some leaderless bacteriocins, including aureocins A53 and A70, and lacticin Q and Z (Coelho et al., 2014; Iwatani et al., 2013, 2012; Nascimento et al., 2012; Netz et al., 2001). An ABC-type multi-drug resistance transporter protein has been described to play a role in the secretion and immunity of the leaderless bacteriocin, LsbB (Gajic et al., 2003), however a common consensus for the production of leaderless has yet to be discovered. The structural genes and genetic determinants of leaderless bacteriocins can be located on the chromosome or plasmid of the producing strains (Perez et al., 2018). For example, the structural gene and biosynthetic genes of weissellicin Y and M, and enterocin MR10 appear to be located on the chromosome of the producing strains, *Weissella hellenica* QU13 (Masuda et al., 2016, 2012) and *Enterococcus faecailis* MRR-10-3 (Martín-Platero et al., 2006), respectively. Whereas the structural genes and genetic determinants of aureocins A53 and A70 (Netz et al., 2002c, 2001), epidermicin NI01 (Sandiford and Upton, 2012), lactolisterin BU (Lozo et al., 2017), and lacticin Q and Z (Iwatani et al., 2007a, 2012, 2013) are plasmid-encoded. However, the specific function of the genes within the biosynthetic clusters remains ambiguous.

Generally, the production of class II leaderless bacteriocins requires four genes (Perez et al., 2018), including (1) a structural gene, (2) an immunity gene, (3) an ABC-type transporter, and (4) an accessory protein, which has been taught to encode an ABC

transporter protein complex that improves peptide production and self-immunity (van Belkum et al., 2010) (see Figure 3). Although genes encoding proteins, including histidine kinases and transcriptional regulators, which are involved in the regulation of class II bacteriocins have been identified within the bacteriocin operon, some class II bacteriocins have been found to be regulated by temperature (Enterocins L50A and B, (Criado et al., 2006)) and nutrient availability (weissellicin Y, (Masuda et al., 2016)).

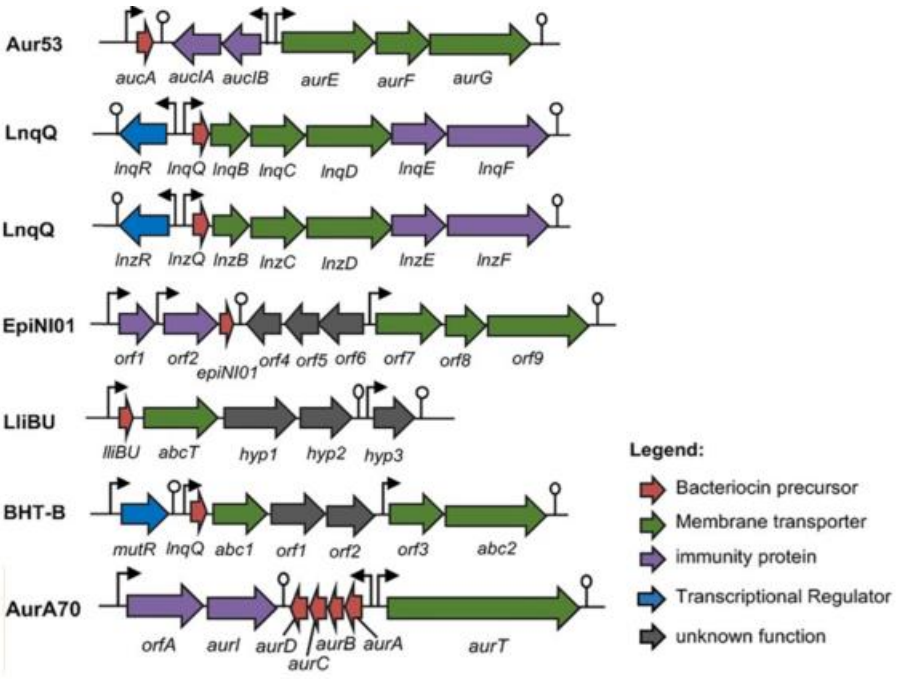

Figure 3. Biosynthetic gene cluster of leaderless bacteriocins. Depicting the single peptide bacteriocins, including aureocin A53, lactocin Q and Z, Epidermicin NI01, Lactolisterin BU and BHT-B, and the multi-peptide bacteriocin aureocin A70. Genes are coloured according to putative function. The putative promoter regions are indicated using arrows, and lollipop symbols represent putative terminators. Taken from Perez et al. (2018).

There appears to be no common mechanism for the regulation of the production of leaderless bacteriocins (Perez et al., 2018). For lacticin Q, a transcriptional regulator (LnqR), was shown to positively affect the transcription of its biosynthetic genes, and subsequently improving bacteriocins production (Iwatani et al., 2013). For weissellicin M and Y, produced by *W. hellenica* QU13, bacteriocin production was based on nutritional conditions (Masuda et al., 2016). The presence of thiamine enhances bacterial growth, however significantly decreases the production of weissellicin Y, however the production of weissellicin M remains uneffected (Masuda et al., 2016). The production of enterocin L50 and enterocin Q, produced by *E. faecium* L50, were shown to be temperature regulated (Masuda et al., 2016). Enterocin L50A and B were synthesized at 16 – 32^{o}C, but production was significantly reduced beyond 37^{o}C, whereas for enterocin Q, bacteriocin production occurred within the temperatures of 16 – 47^{o}C (Criado et al., 2006).

A common structural motif has been identified among leaderless bacteriocins, referred to as a saposin fold. A saposin fold is the structural confirmation of amphipathic α-helices organised within a distinct shape, resulting in a high concentration of hydrophobic residues at the core (Ahn et al., 2006). Unexpectedly given the differences in amino acid sequence length and homology, the majority of leaderless bacteriocins display the characteristic saposin fold (Acedo et al., 2016). This structural conformation has been identified in numerous heterologous and unrelated peptides (Perez et al., 2018). However, unlike native saposins and related peptides, which contain intracellular disulphide bridges between cysteine residues, leaderless bacteriocins lack disulphide bridges to provide stability to the overall structure. These intramolecular bridges play a significant role in providing overall stability to the peptide. For leaderless bacteriocins,

the stability of fold occurs through hydrophobic and electrostatic interactions of the peptide (Towle and Vederas, 2017). Additionally, this structural formation is thought to play a key role in the ability of the peptide to interact with target membrane, and elicit their antibacterial activity (Ahn et al., 2003). As mentioned previously, the majority of leaderless bacteriocins elicit their antimicrobial action by interfering with the bacterial membrane (Perez et al., 2018). These saposin structural confirmations are thought to strongly influence the interaction of leaderless bacteriocins with surface of the bacterial membrane via electrostatic interaction, and their hydrophobic nature plays a key role in pore formation (Towle and Vederas, 2017). However, the leaderless bacteriocin LsbB, produced by *Lactococcus lactis* subsp. *lactis* BGMN1-5 (Kojic et al., 2006), displays no saposin fold conformation, and therefore, should not be associated with the saposin structure. Alternatively, LsbB and other similar bacteriocins including enterocins K1, Q, and EJ97, are comprised of a single α-helical structure located at the N-terminal region, and instead of saposin fold, the C-terminal region remains unstructured (Ovchinnikov et al., 2014).

1.12 Mode of action of leaderless bacteriocins

As previously mentioned, the aureocin-like group of antimicrobial peptides are cationic and tryptophan-rich (Netz et al., 2002b). The tryptophan residues are thought to play a significant role in their ability to interact with the target membrane (Fimland et al., 2002). Mostly, the antimicrobial activity of leaderless bacteriocins have been demonstrated to not be dependent on a target receptor (Perez et al., 2018). With regard mode of action studies, lacticin Q is perhaps the most characterised. Lacticin Q displays a very potent antibacterial activity (Fujita et al., 2007). This strong bioactivity can be attributed to its ability to form toroidal pores in the bacterial membrane, which allow the

efflux of intercellular components, and subsequently cell death (Yoneyama et al., 2009). Pore formation occurs through the electrostatic interaction of the positive lacticin Q molecule with the negatively charged bacterial cell membranes. The cationic lacticin Q peptide quickly interferes with the phospholipid bilayer causing pore formation combined with lipid "flip-flop". However, the pores formed are short-lived, once the lacticin Q peptide has translocated beneath the bacterial membrane, they begin to close (Yoneyama et al., 2009). However, despite not requiring a receptor molecule, the antibacterial activity of lacticin Q is still highly dependent on the physiochemical characteristics of the target membrane, therefore resulting in selective inhibition (Yoneyama et al., 2011). Surprisingly, lacticin Q exhibits a very selective antimicrobial activity. This selective inhibition can be attributed to its affinity to the target cellular membrane (Perez et al., 2018). Nevertheless, lacticin Q remains a very strong bioactive peptide which exhibits potent bioactivity against a number of Gram-positive bacteria, including *S. aureus* (Fujita et al., 2007).

1.13 Class III – CoNS bacteriocins

These bacteriocins are defined as structurally large and heat labile molecules (Cotter et al., 2005). Traditionally, class III bacteriocins are large (>30 kDa), heat labile proteins. The members of Class III bacteriocins can be further separated into groups; Class III (A) bacteriolytic proteins (or bacteriolysins), inhibit target strains by lysing the cell well, and Class III (B) non-bacteriolytic antimicrobial proteins (Helveticin J produced by *Lactobacillus helveticus* 481) (Collins et al., 2017). Many different Gram-positive bacteria have shown to produce bacteriolysins, however the staphylococcal-derived lysostaphin is considered to be the most studied and characterised with regard to its clinical applications (Bastos et al., 2010).

1.13.1 Lysostaphin

This Class III bacteriolytic enzyme is produced by *S. simulans* biovar *staphylolyticus* (designated K-6-WI) (Schindler and Schuhardt, 1964), and was probably the first staphylococcin to be discovered. Lysostaphin consists of 246 amino acid residues and has a confirmed molecular weight of 26,926 Da (see Table 1). Initially, lysostaphin is produced as a preproenzyme containing 493 amino acids, which is transported via a leader sequence (Heinrich et al., 1987). Notably, the prolysostaphin protein exhibits 4.5-fold less antimicrobial activity, when compared with the fully-activated lysostaphin molecule (Thumm and Götz, 1997)(see Figure 4). The lysostaphin protein is comprised of two distinct domains, (1) an N-terminal endopeptidase domain required for proteolytic activity, and (2) a C-terminal domain involved in binding and directing lysostaphin towards bacterial the cell wall (Baba and Schneewind, 1996; Schindler and Schuhardt, 1964).

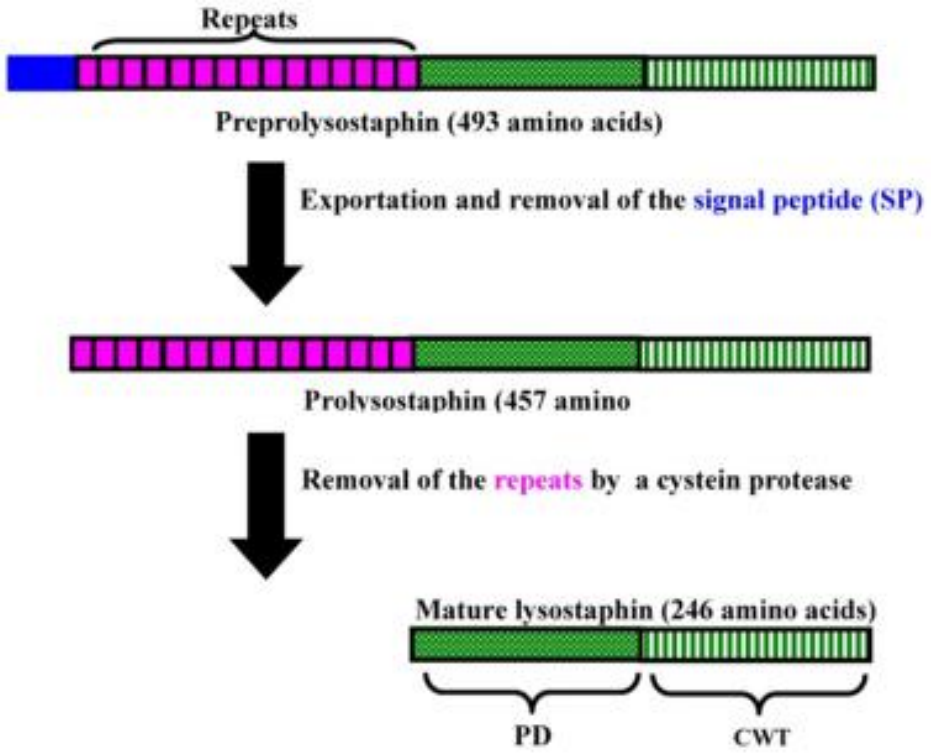

Figure 4. Lysostaphin synthesis. Lysostaphin is initially synthesized as a preproenzyme, and after the removal of the leader sequence (Illustrated in blue (SP)), prolysostaphin is transported out of the cell. Proteolytic cleavage of the propeptide produces the active lysostaphin molecule. The two domains of lysostaphin are displayed, the N-terminal peptidase domain (PD) responsible for proteolytic activity and the C-terminal wall targeting domain (CWT), which directs and binds lysostaphin to the target receptor on the cell surface. Taken from (Bastos et al., 2009).

1.13.2 ALE-1

A homolog of lysostaphin has been described and characterised by Sugai and colleagues (Sugai et al., 1997a), a staphylolytic enzyme called ALE-1 is produced by *S. capitis* EPK1. The molecular structure of ALE-1, mode of action and self-immunity mechanisms, are similar to those described for lysostaphin (Sugai et al., 1997a, 1997b). Similar to lysostaphin, ALE-1 is staphylolytic enzyme, which consists of 362 amino acid residues (Sugai et al., 1997a), which targets the glycyl-glycine bonds in the interpeptide bridges of the peptidoglycan. The activate ALE-1 molecule with a confirmed molecular weight of 35, 600 Da, is obtained following the removal of the preenzyme after the alanine at position 35 (Sugai et al., 1997a). However, unlike lysostaphin, the N-terminal repeat domain of ALE-1 does not undergo the proteolytic cleavage.

1.14 Mode of action of Class III bacteriocins

The peptidoglycan layer plays an important role in the bacterial cell wall, and has multiple functions, including providing the cell with strength, rigidity and shape, protection against osmotic pressure, and allows cell growth and division to occur (Martinez de Tejada et al., 2012). Lysostaphin is a bacteriolytic protein, and elicits its killing effect by degrading the outer membrane of target bacteria, including *Staphylococcus* strains (Bastos et al., 2010). The breakdown of the bacterial cell-wall can be attributed to the proteolytic cleavage of glycyl-glycine residues (see Figure 5). Lysostaphin has been reported to display potent anti-staphylococcal activity primarily against CoNS and CoPS strains, however increased lysostaphin concentrations and incubation times are required to inhibit some CoNS strains, like *S. epidermidis* (Bastos et al., 2010; Zygmunt WA, Browder HP, 1968). The antimicrobial activity of lysostaphin is highly dependent on its proteolytic ability to cleave and disrupt the peptidoglycan layer

of target bacteria (Bastos et al., 2010). Lysostaphin targets the penta-glycine cross-bridge of the peptidoglycan, which consists of five glycine residues staphylococcal species (Gründling and Schneewind, 2006). More specifically, the proteolytic activity of lysostaphin cleaves between the third and the fourth glycine residues (see Figure 5). Staphylococcal strains exhibiting lysostaphin resistance, contain a higher amount of serine residues in the penta-bridge (Kumar, 2008).

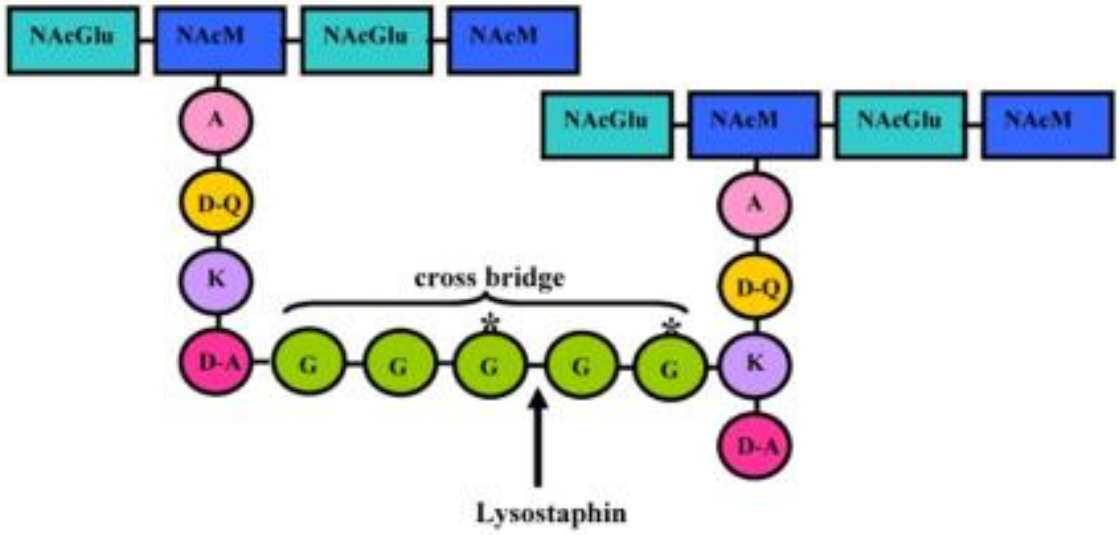

Figure 5. Peptidoglycan structure of *Staphylococcus aureus*, and the lysostaphin proteolytic cleavage site within the staphylococcal peptidoglycan. Black arrow indicates the specific site proteolytic cleavage, between the third and fourth glycine residues. (NacGlu, Acetylglucosamine; NacM, Acetylmuramic acid; A, Alanine; D-Q, Glutamine; K, Lysine; D-A, Alanine; G, Glycine). Taken from (Bastos et al., 2010).

1.15 Class IV or cyclic bacteriocins

These peptides are head to tail cyclysed (occurs through bonding between C to N terminus) to assume their distinctive structure (Arnison et al., 2013; Gabrielsen et al., 2014). All members of Class IV bacteriocins are produced by Gram-positive bacteria, and share common characteristics, which has allowed for them to be sub-divided into two groups (Gabrielsen et al., 2014). The first sub-group contains peptides that possess a high isoelectric point and are positively charged peptides while preserving the overall cationic charge (Arnison et al., 2013; Gabrielsen et al., 2014). The second sub-group contains peptides with a low isoelectric point, and are negatively charged (Arnison et al., 2013; Gabrielsen et al., 2014).

Class IV antimicrobial peptides are initially produced as linear precursor proteins, containing a leader sequence (2–35 amino acids) that is removed during the maturation process (Van Belkum et al., 2011). The linear peptide (35–70 aa residues) is cyclized through the formation of a peptide bond between the N and C terminus, before being transported out of the cell. The enzymes responsible for removing the leader sequence and ligation of the carboxy and amino termini have yet to be identified (Potter et al., 2014). The cyclic nature of these peptides provides them with proteolytic resistance and temperatures stability (Potter et al., 2014). Several genetic determinants are involved in production of cyclic bacteriocins (van Belkum et al., 2011). Although the number of genes among the gene clusters varies and similarity between the gene products of the various gene clusters is limited, they share a number of features. Most of the proteins encoded by these gene clusters contain multiple putative membrane-spanning domains (van Belkum et al., 2011).

1.15.1 Aureocyclicin 4185

Aureocyclicin 4185 is a Class IV cyclic bacteriocin, produced by bovine mastitis isolate *S. aureus* 4185, and is encoded on plasmid pRJ101 (Francisco et al., 2017; Potter et al., 2014). Aureocyclicin 4185 is a cationic peptide, contains a high quantity of hydrophobic residues, and so, is a Class I sub-group of cyclic bacteriocins (Potter et al., 2014; Van Belkum et al., 2011). Aureocyclicin 4185 consists of 60 amino acid residues, and a confirmed molecular weight of 5607 Da (Potter et al., 2014). The cyclic peptide could not be detected from the culture supernatant, and MS analysis of bioactive HPLC fractions were unable to detect the 5607 Da peptide, suggesting that aureocyclicin 4185 is produced at very low concentrations (Ceotto et al., 2010a; Potter et al., 2014). The aureocyclicin 4185 gene cluster (consisting of *acl*X, *acl*B, *acl*I, *acl*T, *acl*C, *acl*D, *acl*A, *acl*F, *acl*G and *acl*H) is similar to that of carnocyclin A, a Class IV bacteriocin produced by *Carnobacterium maltaromaticum* UAL307 (Martin-Visscher et al., 2008; Potter et al., 2014).

1.16 Potential Applications of Staphylococcal-derived bacteriocins

Bacteriocin production among bacteria is thought to play an ecological role, and it has been proposed that their primary role is provide the producing strain with a competitive advantage against rivalling bacteria within the same ecological niche (Dobson et al., 2012). The antagonistic activity of these producing strains may hold significant biotechnological potential. Some staphylococcal-derived bacteriocins are potent antimicrobials, capable of suppressing the growth of several notable pathogens like MRSA (Sandiford and Upton, 2012) and *L. monocytogenes* (Fagundes et al., 2016). Therefore, demonstrate potential for use within the food industry, as natural bio-

preservatives, or as therapeutics to treat bacterial infections. Throughout the years, most academic research groups have investigated LAB bacteriocins for their attractive properties relating to the use as natural bio-preservatives in food products (Cotter et al., 2005). In 1988, nisin received approval from the US Food and Drug Administration (FDA) and acquired a generally regarded as safe (GRAS) designation for the safe use in food products (Chen and Hoover, 2003; Chikindas et al., 2018; Paul D Cotter et al., 2005). However, despite the global research, and the continuous isolation of novel antimicrobials, only nisin has earned FDA approval for use as food bio-preservative. Moreover, in recent years there has been an ever-growing number of scientific papers been published highlighting the potential of staphylococcal-derived bacteriocins to combat bacterial infections (Bastos et al., 2009; Bonelli et al., 2006; Fagundes et al., 2016; Fujita et al., 2007; Sandiford and Upton, 2012). However, despite the numerous studies, only nisin has been given approval (Dicks et al., 2018). The diversity among bacteriocin-producing bacteria and the abundance of literature/studies highlighting their potency in several applications, makes staphylococcins favourable alternatives to antibiotics.

1.17 Food preservation

Utilizing the competitive nature of bacteriocin producing *Staphylococci* has practical applications within the food industry. Many LAB producing strains that inhibit the growth of various food-borne pathogens have been commercially investigated for their potential as natural food bio-preservatives. Most of the bio-preservative methods involving LAB antimicrobial peptides can be divided into three groups: (1) Partially purified bacteriocins, such as Nisaplin® which contains 2.5% nisin A, dairy and other food-grade fermented products containing bacteriocins in the form of a crude fermentate

(Field et al., 2015b), (2) the MicroGARD® series of bacteriocin-containing products, and (3) bacteriocin-producing starter cultures (Mills et al., 2017).

Staphylococcins exhibit potential for use as food preservatives. For instance, aureocin A53 and A70 demonstrated several key features which make them promising candidates for use as food preservatives in dairy products (Carlin Fagundes et al., 2016; Fagundes et al., 2016). Both aureocin peptides demonstrated potent anti-listerial activity, efficiently controlling and significantly reducing cell counts of *L. monocytogenes*. Aureocin A70, at a concentration of 16 AU/ml of partially purified peptide, was revealed to be bactericidal against the food-borne *L. monocytogenes* L12 strain (starting inoculum 4-log CFU/mL) when tested in skimmed milk, and caused a significant 5.51-log decrease in viable cell counts following 7 days of incubation (Carlin Fagundes et al., 2016). Aureocin A70 demonstrated no cytotoxic effects against eukaryotic cell lines, nor displayed any haemolytic activity against blood cells (Carlin Fagundes et al., 2016). Carlin Fagundes and colleagues (Carlin Fagundes et al., 2016) revealed key attributes of aureocin A70 considered important for bio-preservation, the remarkable stability of aureocin A70 during storage at different temperatures, which remained stable for 4 weeks at 25°C, 16 weeks at 4°C and 20 weeks at -20°C. Additionally, when treated with simulated gastric juice, a 84.4% reduction in antimicrobial activity was observed following 90 minutes of treatment, therefore unlikely to have a negative impact on the gut microbiota (Carlin Fagundes et al., 2016). Furthermore, aureocin A70 remained active when adsorbed to a polyethylene plastic surface proposing that aureocin A70 may be incorporated into the food packaging to provide an additional protective barrier to control the growth of undesirable bacteria in food products (Carlin Fagundes et al., 2016).

Additionally, a study by Fagundes and colleagues (Fagundes et al., 2016) showed the Class II aureocin A53 to be highly potent against food-borne *L. monocytogenes.* The anti-listerial activity of aureocin A53 was examined in a skimmed milk food model against food-associated *L. monocytogenes* L12 strain (starting inoculum 4-log CFU/mL) and stored at 4°C. The staphylococcin, at a concentration of 256 AU/ml, reduced listerial counts by 7.7-log units following 7 days of treatment (Fagundes et al., 2016). Similar to aureocin A70, aureocin A53 demonstrated no cytotoxic effects or haemolytic activity (Fagundes et al., 2016). During susceptibility resting, aureocin A53 remained stable for 4 weeks at 25°C, 48 weeks at 4°C and 72 weeks at -20°C (Fagundes et al., 2016). Moreover, aureocin A53 proved to be sensitive to gastric juice and bile salts, indicating that it should not have a negative impact on the gut microbiota (Fagundes et al., 2016; Gálvez et al., 2007). A study by Varella Coelho and colleagues highlighted a synergistic relationship between aureocins A53 and A70 against 239 clinical bovine mastitis strains, including 165 *Staphylococcus aureus* strains and 74 *Streptococcus agalactiae* strains (Varella Coelho et al., 2007). Moreover, this synergistic relationship between the aureocin peptides increased the spectrum of strains inhibited, and was confirmed using agar-based well diffusion assays, and different combinations of the partially purified aureocins (Varella Coelho et al., 2007). The production of non-antibiotic preparations based on combinations of aureocins A53 and A70 represent an natural alternative for use as a food bio-preservative.

S. hyicus 3682 produces a lantibiotic named hyicin 3682, which is similar to epidermin (Fagundes et al., 2011). Using deferred antagonism assays, hyicin 3682 was shown to inhibit the food-borne pathogen *L. monocytogenes* (Fagundes et al., 2011). Hyicin 3682 revealed important characteristics which make it a potential candidate as

natural food biopreservative. Although hyicin 3682 is susceptible to high temperatures (80°C - 121°C), it has potential for use as biopreservative of raw or minimally processed foods stored at low temperatures (Fagundes et al., 2011). Additionally, hyicin 3682 was capable of maintaining full antimicrobial activity at low and neutral pH values (Fagundes et al., 2011). However, when assessed at higher pH values 9 and 11, hyicin 3682 bioactivity decreased by 75% and 87.5%, respectively (Fagundes et al., 2011). Therefore, this staphylococcin has the potential for use as a food bio-preservative for acidic foods.

The lantibiotic warnericin RB4, produced by *S. warneri* RB4, represents another promising staphylococcin for use as a natural food biopreservative. (Minamikawa et al., 2005). The staphylococcal-derived lantibiotic was capable of inhibiting *Alicyclobacillus acidoterrestris*, a thermoacidophilic spore-forming Gram-positive bacteria that frequently causes contamination of fruit juices and other beverage products (Tianli et al., 2014). Minamikawa and colleagues (Minamikawa et al., 2005) revealed, through agar-based well diffusion assays, that warnericin RB4 had a narrow spectrum of activity, inhibiting only *Alicyclobacillus acidoterrestris*, *A. acidocaldarius*, and *Micrococcus luteus*. Warnericin RB4 demonstrated main key features, including a specific bioactivity against a problematic food-spoilage thermoacidophilic bacteria and its optimal pH range of 2 - 6, making it an ideal candidate for use as a bio-preservative in fruit juices, acidic juices and other beverage products within the juice industries (Minamikawa et al., 2005; Tianli et al., 2014).

While the majority of staphylococcins are produced by strains that are strongly linked with pathogenicity, frequently reported as causative agents of opportunistic infections (Otto, 2010) and display a resemblance with haemolytic peptides, these potent

Class II antimicrobial peptides have demonstrated no cytotoxic or haemolytic activities, highlighting their safe use in food products. Additionally, both bacteriocins possess attractive characteristics required for food preservation, including pH and temperature stability and retaining bioactivity in high salt concentrations (Carlin Fagundes et al., 2016; Fagundes et al., 2016; Nascimento et al., 2004; Netz et al., 2002c). The biotechnological potential of leaderless bacteriocins can be attributed to the "simplistic" structure of these antimicrobial peptides. The commercial application of leaderless bacteriocins has generated much interested from numerous industries, because they display potent antibacterial activity, have no leader sequence, require no post-translational modifications and the structural gene can be introduced into prokaryotic or eukaryotic recombinant expression systems. In fact, to avoid disapprovals related to bacteriocin production by a pathogenic bacteria, the heterologous expression of the aureocin A70 gene cluster located on plasmid pRJ6 (Netz et al., 2001) in food-grade Staphylococcal strains is underway. *Staphylococcus carnosus* and *Staphylococcus xylosus* are frequently used within the food industry, and have been designated as non-pathogenic (Löfblom et al., 2017), are being used as expression systems for the pRJ6 plasmid to ensure food safety (Carlin Fagundes et al., 2016).

1.18 Clinical/Medical applications

Currently, the rapid development of antibiotic resistance among bacteria represents a major threat to global public health. The rapid emergence of antibiotic-resistant bacteria has urged microbiologists and infectious-disease scientists to consider novel alternative antimicrobial strategies with unique modes of action against these problematic pathogens. Despite warnings about their overuse, antibiotics remain among the most prescribed drugs worldwide, which contribute to the disturbing rate at which

strains become resistant. The CDC estimates that 50% of the prescribed antibiotics are not necessary, or are not the appropriate course of treatment for the infection (CDC, 2019; Milani et al., 2019). In the United States, doctors continue to prescribe antibiotics, with 22.0 standard units (a unit corresponding to one dosage, i.e. one pill, capsule, or ampoule) of antibiotics prescribed per person (Van Boeckel et al., 2014; Ventola, 2015), which leads to bacteria developing resistance to several antibiotics (Thomsen, 2016). Therefore, it is becoming increasingly urgent to identify and develop novel antimicrobial agents, and bacteriocins have become an attractive alternative and should be considered as an important source of antimicrobial compounds. Thus far, the majority of the previously described staphylococcal-derived bacteriocins have displayed potent antimicrobial activity against several important medical and veterinary pathogens.

1.18.1 Inhibition of Staphylococcal biofilms

Critically, antibiotic resistance coupled with the ability to form biofilms contribute to the ability of *S. aureus* to invade and persist within healthcare environments (Flemming and Wingender, 2010; Otto, 2008). Biofilm formation is recognized as a significant virulence factor, and represents a major hurdle in the treatment of *Staphylococcus* infections (Otto, 2008). In particular, the opportunistic pathogens *S. aureus* and *S. epidermidis* are the main causative agents for nosocomial infections and medical devices infections, which typically involve biofilms (Khatoon et al., 2018; Otto, 2008). Considering the increased virulence and antibiotic-resistance of strains, like MRSA, the effective treatment of persistent biofilm infections is significantly delayed (Gould et al., 2012). Staphylococcal-derived lantibiotics, including epidermin and gallidermin, represent a group of promising therapeutic treatment alternatives, capable of prevent MRSA biofilms, by interfering with cell wall biosynthesis (Götz et al., 2014). In

fact, gallidermin is capable of inhibiting the growth (MIC values of 4 - 8 μg/ml), and also effectively prevented the formation of *S. aureus* and *S. epidermidis* biofilms, and at sub-lethal peptide concentrations (Saising et al., 2012). The ability to gallidermin to prevent biofilm formation might be due to suppression of biofilm-associated genes such as *ica* and *alt* (required for adhesion and major autolysin, respectively) (Saising et al., 2012). Contrastingly, incomparsion to other staphylococcal biofilm treatments which require high peptide concentrations, gallidermin is capable of preventing biofilm formation at peptide concentrations (0.5 μg/ml) that are sub-inhibitory for growth (Saising et al., 2012). The exposure of staphylococcal biofilms to sub-inhibitory concentrations of antibiotics such as erythromycin, vancomycin, tetracycline, nafcillin and furanone, has been demonstrated to enhance biofilm formation (Yu et al., 2018).

1.18.2 Inhibition of pathogenic *Staphylococcus* from medical devices

Recent clinical studies have demonstrated that use of medical devices in medicine is constantly increasing, and plays a pivotal role in human healthcare (Zheng et al., 2018). Despite the increased hygiene and sterilization strategies utilized by hospitals of medical devices, bacterial infections still occur. Among Gram-positive bacteria, *S. aureus* is leading causative agent of medical device infections (Zheng et al., 2018). In the United States, its estimated that 80,000 cases of bloodstream infections occur each year as a result of contaminated catheters (Gominet et al., 2017). Therefore, the need for a protective antimicrobial surface agent to prevent the colonization of *S. aureus* or *S. epidermidis* is urgently required. In 2006, a study by Fontana and colleagues (Fontana et al., 2006) revealed that the staphylococcal-derived lantibiotics Pep5 and epidermin, were capable of preventing the colonisation of *S. epidermidis* on the surface of silicone catheters. Moreover, the lantibiotics caused a significant reduction (90%) in the bacteria able to

adhere to the silicone catheter. Additionally, these results are even more impressive considering treatment with vancomycin alone caused a 32% bacterial reduction, and when used in combination with rifampicin resulted in a 42-81% reduction (Raad et al., 1995).

An investigation conducted by Wu and colleagues (Wu et al., 2003) has demonstrated the effectiveness of lysostaphin as an antibacterial surface agent in preventing *S. aureus* and *S. epidermidis* colonization. This study indicated that lysostaphin displayed highly potent specific activity against *S. aureus* cells, and *in vitro* studies showed that lysostaphin could disrupt *S. aureus* biofilms, on different materials, at peptide concentrations as low as 1 µg/mL (Wu et al., 2003). In another related study, Shah and colleagues, (Shah et al., 2004), established the efficiency of lysostaphin to decrease the occurrence and colonisation of *S. aureus* on different surfaces. Lysostaphin-coated wells of a twenty-four well polystyrene plates resulted in a 99.5% reduction in bacterial counts. Additionally, lysostaphin-coated catheters were able to retain their antibacterial activity for at least four days after coating (Wu et al., 2003). These studies demonstrate that the staphylococcal-derived lantibiotics, epidermin and gallidermin, and Class II bacteriocin, lysostaphin, can be effective antimicrobial surface agents, displaying potent antimicrobial activity, capable of adhering to and preventing *S. aureus* colonisation on plastic surfaces and may serve to provide protection against staphylococcal colonization of catheters. However, it's worth noting that no antimicrobial surface agent is 100% effective at preventing *S. aureus* colonisation.

1.18.3 Prevention of colonisation of *Staphylococcus aureus*

In addition to biofilm formation, the colonisation of *S. aureus* in the nasal carriage is a major risk factor of bacterial infection following surgical procedures (Bode et al., 2010; Lee et al., 2015; Muñoz et al., 2008). Additionally, the substantial mortality rate

associated with *S. aureus* infections, can be attributed to methicillin-resistant strains, MRSA (Hassoun et al., 2017; Klein et al., 2007). MRSA can be part of the normal body flora, especially in the nose, causing severe infection, especially in patients with extended hospital stays or underlying disease, or after antibiotic use (Bradley, 2015). Currently, mupirocin is the antibiotic of choice for the elimination of *S. aureus* nasal colonization, however the rate of resistance to this antibiotic is increasing (Antonov et al., 2015; Desroches et al., 2013). While nisin and other naturally-occurring variants have been reported to be effective against MRSA (Field et al., 2016; Shin et al., 2016), staphylococcins, namely epidermicin NI01, has been shown to be an efficient inhibitor of MRSA strains. Epidermicin NI01 has demonstrated antimicrobial activity against MRSA in the nano-molar range (MIC values of 1-2 µg/ml – 160- 329 nM) (Sandiford and Upton, 2012). In 2017, Halliwell and colleagues (Halliwell et al., 2017) examined the effectiveness of epidermicin NI01 to decolonization MRSA from the nares of rats, using single- and multiple-applications. While the multiple-dose of epidermicin NI01 did not have a significant impact, a single application of epidermicin NI01 (0.8%) was sufficient in eradicating MRSA, when administered twice daily for 3 days (Halliwell et al., 2017).

In a similar study, Kokai-Kun and co-workers (Kokai-Kun et al., 2003) examined the ability of three different antimicrobial compounds, nisin (Class I – lantibiotic) lysostaphin (Class III – bacteriocin), mupirocin (topical antibiotic), to eradicate MRSA from the nares of a rat model. A single application of lysostaphin (approximately 150 µg/ml) significantly decreased the *S. aureus* nasal colonization in 93% of rat models, after 4 hours. For mupirocin, three applications over three days was required to reduce *S. aureus* nasal colonisation. However, even at high concentrations, the potent lantibiotic nisin (approximately 1.5 mg) was unable to reduce staphylococcal nasal colonisation in

this model (Kokai-Kun et al., 2003). Collectively, these studies demonstrate the effectiveness of staphylococcal bacteriocins to control and eradicate *S. aureus* colonisation, and have been established to more potent than conventional antibiotics.

1.19 Bovine Mastitis

Globally, bovine mastitis is considered to be one of the most common diseases causing high financial losses in dairy industries (Käppeli et al., 2019). *Streptococcus* spp. have been shown to be susceptible to penicillin, which is the antibiotic of choice for bovine mastitis treatment in cases caused by *Streptococcus* spp. and *Staphylococcus* spp. including *S. aureus* (Käppeli et al., 2019). The increasing number of strains developing resistance as a result of the extensive use of antibiotics in human and veterinary medicine worldwide has generated the search for antimicrobial alternatives (Bastos et al., 2009; Käppeli et al., 2019). Alternative safe antimicrobial agents, such as staphylococcins, to treat bovine mastitis would be very beneficial to the dairy industry. However, nisin remains the only bacteriocin to have received FDA approval for use as a bioactive agent in two commercial products Wipe-Out, and MastOut (Cotter et al., 2005; Pieterse and Todorov, 2010). Coelho and colleagues (Varella Coelho et al., 2007) investigated the specific bioactivity of seven staphylococcal-derived bacteriocins, including aureocins A53, A70, 215FN and the lantibiotics; Pep5, epidermin, epilancin K7 and epicidin 280, against 239 bovine mastitis strains (*S. aureus* (165 strains) and *S.* agalactiae (74 strains)). This study revealed the effectiveness of staphylococcins against bovine mastitis strains, with epidermin and aureocin A53 able to inhibit 89% and 77% of all strains tested, respectively (Varella Coelho et al., 2007). In the case of *S. aureus* alone, aureocin A53 and Pep5 inhibited 91% and 63% of strains, respectively. Furthermore, this study identified a synergistic relationship between aureocins A70 and A53, with the peptide

combination displaying an enhanced activity spectrum by inhibiting 93% of all strains tested. This synergistic relationship between the aureocin A53 and A70, is fascinating considering, when taken independently, the bacteriocins were only capable of inhibiting 68% and 1.4% of the streptococcal strains, respectively (Varella Coelho et al., 2007). The concentration of each peptide required for the combined antimicrobial activity was 20 AU/ml. It has been purposed that using bacteriocin combinations can result in synergistic activity which can dramatically improve their antibacterial activity and activity spectrum against target bacteria (de Freire Bastos et al., 2015; Nascimento et al., 2006). Additionally, this study highlights the combination of Pep5 and aureocin A53, which increased their anti-staphylococcal activity and spectrum of the staphylococcal strains inhibited (Varella Coelho et al., 2007). Unlike traditional antibiotics, the majority of staphylococcins elicit their killing action by targeting the bacterial membrane, resulting in the efflux of cell components and death (Bastos et al., 2009; Newstead et al., 2020). Thus, the use of staphylococcin combinations represent a promising approach to control and prevent the increase of resistant bacteria in veterinary applications. Also, the use of bacteriocins with different modes of action, will make it difficult for the bacteria to acquire resistance to the antimicrobials.

1.20 Conclusions and Future prospects

The global overuse and misuse of antibiotics has led to rise of multi-antibiotic resistant bacteria. Indeed, the increasing threat that antimicrobial resistance poses, coupled with the diminishing antibiotic discovery rates, has forced the immediate search for antimicrobial alternatives. Recent bacteriocin isolation studies have emphasised the wealth of natural antimicrobial compounds that are present in the microbial world and highlight their continuing potential for development as novel antibiotic alternatives.

Certainly, bacteriocins serve as bactericidal agents that can provide us with, at least in part, a solution to this crisis.

Staphylococcal-derived antimicrobial peptides represent a diverse, and a steadily growing, group of antimicrobial compounds. Overall, they have been shown to be effective against various problematic pathogens. Consequently, they possess enormous potential for use in food, medical and veterinary applications. The potent antibacterial potential of staphylococcins is, among other qualities, the most favourable to food, human and animal infectious diseases (Bastos et al., 2010; Braem et al., 2014; Halliwell et al., 2017). The use of staphylococcal-producing strains to treat or limit the spread of infectious diseases appears to be worthwhile and profitable, as they display no cytotoxic activity. Additionally, the effectiveness of staphylococcins has been established against many important pathogens associated with nasal and systemic infections, bacteraemia, bovine mastitis, and listeriosis. Moreover, they demonstrated an ability to prevent biofilm formation and bacterial colonisation on catheter surfaces.

When used in combination with other antimicrobial agents, may allow for the complete removal of harmful chemical substances or antibiotics in food preservation or therapeutic applications. Additionally, the combined use of different antimicrobial agents has advantages over their individual use to control bacterial growth. Unlike traditional antibiotics, the majority of staphylococcins penetrate and/or disrupt the membrane of the target cells, causing a rapid efflux of components and, ultimately, resulting in cell death. Therefore, the combination of traditional antibiotics with bacteriocins represents another approach in combatting the rise of antibiotic-resistant bacteria as it would be hard for bacteria to acquire resistance to different modes of action simultaneously. The synergistic

activity of staphylococcins has been shown to be efficient in preventing the growth of undesirable bacteria, while also increasing the activity spectrum of bacteria inhibited.

Recent improvements in biotechnology have revealed an encouraging, and rapidly growing area of research focused on the bioengineering of antimicrobial peptides. Bacteriocins are gene-encoded, which has allowed for the development of bioengineering approaches that will provide a better understanding into the roles, functions and importance of structure/function relationships. These bioengineering strategies have the ability to create novel staphylococcin mutants with enhanced functionalities, including increased bioactivity, broader activity spectrum, temperature and pH stability, and protease resistance. Over the years, researchers from interdisciplinary fields have bioengineered nisin derivatives that possess improved peptide functional abilities such as antimicrobial activity (Field et al., 2012, 2010b, 2008; Healy et al., 2013), solubility (Rollema et al., 1995; Yuan et al., 2004) diffusion capabilities (Rouse et al., 2012), bioactivity against Gram-negative bacteria (Field et al., 2012) and overcoming proteolytic cleavage caused by nisin resistance protein (NSR) (Field et al., 2019). The identification of variants with enhanced functionalities represents a crucial breakthrough for nisin bioengineering strategies, but also bacteriocins, and confirms that peptide bioengineering can generate enhanced derivatives to have therapeutic potential for human diseases and target problematic pathogens. Undeniably, these bioengineering strategies could be employed on other bacteriocins including staphylococcins. Additionally, for the safe application of staphylococcins as human therapeutics, it will be essential to gain better insight and understanding into their pharmacodynamics and pharmacokinetics, which in relation to bacteriocin research, is significantly under-investigated. Certainly, if staphylococcal-derived bacteriocins are to be competing against traditional antibiotics as

therapeutic alternatives, a greater focus must be placed on research relating to the interactions between drug and hosts, similar to what was achieved for the *Streptococcus mutans* lantibiotic, MU1140 (Ghobrial et al., 2010).

1.21 Nisin

Bacteriocins are ribosomally produced cationic peptides that inhibit or prevent the growth of closely related bacterial species (Cotter et al., 2013). The best known and most characterised is nisin, produced by strains of *Lactococcus lactis* subsp. *lactis* (Arnison et al., 2013; Field et al., 2008), and recently also isolated from *S. capitis* (O'Sullivan et al., 2020). Nisin is a Class I lantibiotic, comprising of 34 amino acids and five lanthionine rings. Nisin serves as a broad-spectrum bacteriocin and is inhibitory against an extended range of Gram-positive bacteria, including *streptococci*, *staphylococcus*, *lactobacilli*, *micrococci* and *Listeria*. As a cationic bacteriocin, nisin initiates it's antibacterial activity effect on sensitive cells, causing rapid death following exposure (Prince et al., 2016). This bactericidal effect can be attributed to the peptides dual mode of action, by interrupting cell wall biosynthesis (through binding Lipid II) and disrupting the cellular membrane (through pore formation)(Shin et al., 2016).

This dual mode of action can be attributed to the presence of two-structural domains, at the N- and C-terminus. The N-terminal rings of nisin, consisting of A, B, and C, are connected to the C-terminal rings (D and E) by a flexible region of three amino acids (N20, M21 and K22) situated between rings C and D (Field et al., 2008; Gross and Morell, 1971) (see Figure 6). It has been established that the A, B and C rings bind lipid II, thus inhibiting cell wall synthesis. This binding improves the ability of the C-terminal domain to form pores in the cell membrane, resulting in the rapid efflux of ions and cellular contents (Field et al., 2008; Wiedemann et al., 2001a). Pore formation occurs

rapidly and cell lysis occurs within seconds after addition of nanomolar concentrations of nisin to a bacterial culture (AlKhatib et al., 2014; Wiedemann et al., 2004). For Gram-negative bacteria, normally resistant to action of nisin, can be sensitized by the addition of chelating agents, these disrupt the outer membrane and allow the lantibiotic to interact with the cytoplasmic membrane (Field et al., 2012).

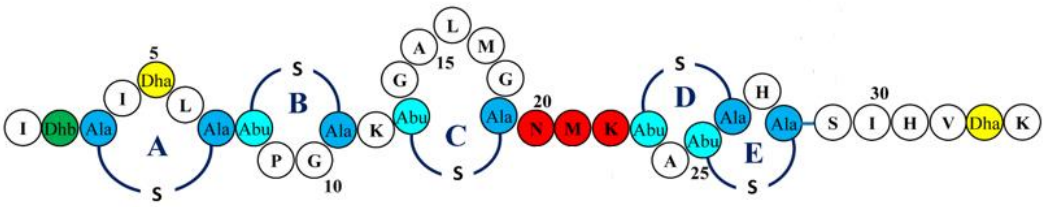

Figure 6. Peptide structure and amino acid composition of nisin A. Residues are represented in the single letter code. Post translational modifications are indicated as follows, Dha: dehydroalanine, Dhb: dehydro butyrine, Abu: 2-aminobutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: 3- methyllanthionine. The nisin hinge region is indicated by red circles (containing asparagine, N20; methionine, M21 and lysine, K22).

Initially, nisin was planned for use as an antibiotic however was deemed unsuitable due to its inability to target Gram-negative bacteria and sensitivity to digestive enzymes and higher pH values. Nevertheless, nisin contains various qualities that make it ideal for use as a food biopreservative, including non-cytotoxic activity, produced by GRAS microorganisms, degraded in the gastrointestinal tract and thus, doesn't negatively effect the gut microbiota (Chikindas et al., 2018; Field et al., 2008). Commercially, nisin is sold under the name of Nisaplin ©, a stable powdered preparation containing

approximately 2.5% nisin (Younes et al., 2017). The control and regulation of foodborne pathogens in food products, using antimicrobial peptides, has been investigated by several research groups over decades (Fagundes et al., 2016; Gálvez et al., 2007; Rouse et al., 2012; Zouhir et al., 2010). However, nisin remains the only commercially available food-grade bacteriocin used as a natural food biopreservative, having FDA approved GRAS status.

Nisin has many applications, but has made a significant impact as a natural bio-preservative in the food industry for different types of foods, including dairy, canned, meat and fish products, alcoholic beverages and salad dressings (de Arauz et al., 2009). LAB possess many attractive attributes which make their bacteriocins highly promising as food biopreservatives: including (1) GRAS status and are viewed as having health benefits, (2) they don't negatively impact the gut microbiota, as they are degraded by digestive proteases, (3) they demonstrated no cytotoxic activity, (4) they retain bioactivity across a range of pH and temperature values, (5) they're highly receptive to bioengineering strategies (Field et al., 2015a, 2019; Rouse et al., 2012; Smith et al., 2016), (6) many LAB bacteriocin producers have different modes of action, and (7) they display potent bioactivity against food pathogenic bacteria, including *L. monocytogenes* and *S. aureus* (Field et al., 2010b, 2015b).

1.22 Naturally occurring nisin variants

The most thoroughly investigated lantibiotic is Nisin A, this pentacyclic antimicrobial peptide consists of 34 amino acids (except nisin U, P and J) residues for which 13 undergo post-translationally modifications. These are mainly produced by *L. lactis* strains (Field et al., 2008, 2015a). Currently, ten naturally occurring nisin derivatives exist, including nisin A, Z, Q, F, U, U2, H, P, O and the most recently

characterised nisin J (O'Connor et al., 2015; O'Sullivan et al., 2020). Nisin exerts its antimicrobial activity through four different activities: 1) through *Nis*K, nisin can regulate its own production, 2) inhibition through pore formation, 3) preventing cell wall biosynthesis, and 4) it prevents the outgrowth of germinated spores (Rink et al., 2007).

1.23 Nisin bioengineering strategies

In recent years, researchers have employed numerous bioengineering strategies with the aim of improving the therapeutic functionalities of antimicrobial peptides, particularly with the lantibiotic nisin. Lantibiotics are gene encoded and thus researchers have sought to exploit this feature by creating novel derivatives. The resulting derivatives were used to explore structure/function relationships of the parent peptide. Frequently, these bioengineering-based approaches have identified peptides with enhanced capabilities, including enhanced antimicrobial activity. Bioengineering strategies can occur in two ways: 1) *in vivo*, where the producing strain is genetically manipulated or using an alternative host to heterologously express the genes, and 2) *in vitro*, using the bacteriocin-associated modification enzymes and biosynthetic machinery outside of a host (Cotter, 2012). In the 1990's, the use of bioengineering strategies to improve lantibiotic peptides began (Kuipers, 1996; Kuipers et al., 1992). Although initial reports provided great insights into structure/function relationship of nisin, no derivatives with enhanced bioactivity were identified (Kuipers, 1996). Although initial bioengineering results were disappointing and did not generate any enhanced variants (Kuipers et al., 1992), the potential benefits of employing this technology were realised.

In 1992, a study by Liu and colleagues (Liu W, 1992) demonstrated the consequences of site-directed mutagenesis on lantibiotics, when it was revealed that a single amino acid change at position four of subtilin (naturally glutamic acid converted

to isoleucine; E4I) resulted in a 57-fold increase in the stability of the peptide. In 2006, the first alanine-scanning mutagenesis was conducted on lacticin 3147 (Cotter et al., 2006). For this study, Cotter and colleagues (Cotter et al., 2006) introduced an alanine residue (or glycine where an alanine was already present) in place of the 59 amino acids, and highlighted specific areas of the peptide where site-specific mutagenesis might generate enhanced variants. Additionally, a site-saturation approach was utilized to bioengineer nukacin ISK-1 (Islam et al., 2009) and mersacidin (Appleyard et al., 2009). In addition to providing great insight into the structure/function relationships of the peptides, these bioengineering studies generated derivatives with enhanced functionalities. For nukacin ISK-1, two derivatives (D13E and V22I) were shown using deferred antagonism assays and MIC determinations to exhibited a two-fold increase in specific bioactivity towards the indicator strains when compared against wildtype (Islam et al., 2009).

Throughout the years, numerous research groups have conducted bioengineering based strategies in an effort to better understand and enhance the functionalities of nisin (Field et al., 2019, 2015c, 2008; Kuipers, 1996). While various bioengineering studies have focused on different sections of the peptide, the N-terminus and hinge region of nisin remain the most frequently studied. The hinge region of nisin is comprised of 3 amino acids (asparagine, N20; Methionine, M21 and Lysine, K22), and is located between the third and fourth rings (see Figure 6). The hinge region of nisin has been studied extensively by many research groups, generating many mutants with enhanced capabilities (Field et al., 2008; Healy et al., 2013; Yuan et al., 2004; Zaschke-Kriesche et al., 2019; Zhou et al., 2015). Yuan and co-workers (Yuan et al., 2004) employed a site-directed approach by incorporating positively or negatively charged amino acids into the

hinge region. Interestingly, this resulted in identification of mutants N20K and M21K which demonstrated enhanced bioactivity against *Shigella flexneri* 51285, *Pseudomonas aeruginosa* 1010 and *Salmonella* 50311 (Yuan et al., 2004). In the study by Field and colleagues (Field et al., 2008), a bank of approximately 8000 random mutagenized nisin variants were screened using deferred antagonism assay against various indicator strains, revealed that the nisin variant K22T demonstrated greater specific bioactivity against bovine mastitis pathogen *S. agalactiae* ATCC 13813, when compared to wild-type. This encouraged field and colleagues (Field et al., 2008) to implement a site-saturation approach for the individual hinge residues which, after combined with a wide range of indicator strains, generated a number of nisin mutants with improved bioactivity. In particular, the derivatives N20P, M21V and K22T were shown using deferred antagonism assays (larger zones of inhibition) and reduced MIC determinations to possess enhanced bioactivity against *L. monocytogenes*, *S. agalactiae* and *S. aureus* (Field et al., 2012, 2010b, 2008)(see Figure 7).

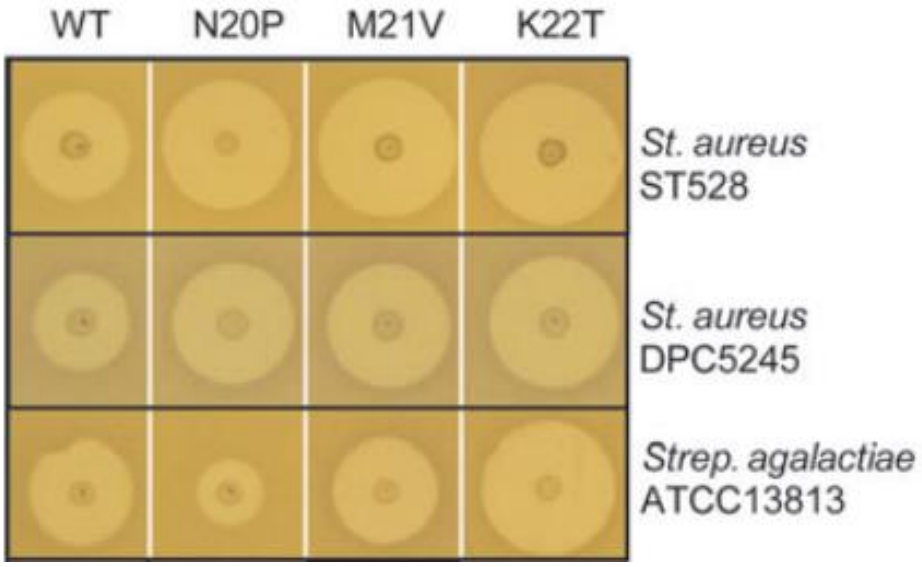

Figure 7. Enhanced bioactivity of nisin hinge mutants. Deferred antagonism assays of the Nisin A (WT), N20P, M21V and K22T mutants against *S. aureus* ST528, *S. aureus* DPC5245 and *S. agalactiae* ATCC13813. Taken from Field et al. (2008).

Another study, conducted by Rink and colleagues (Rink et al., 2007) focused their attention on randomising the N-terminus region of nisin at rings A and B, more specifically at positions 4, 5, 6, 9 and 10. This study highlighted that the functional characteristics of nisin could be improved by altering the amino acids in the N-terminus region. Two nisin derivatives KFI and KSI demonstrated enhanced bioactivity, when compared with wild-type nisin A using IC_{50} determinants against *Leuconostoc mesenteroides, Lactobacillus johnsonii* and *Lactoccus lactis*. Other noteworthy variants include KFI and VFG, which capable of inhibiting the outgrowth of *Bacillus subtilis* 168 spores when investigated in a growth assay in microwell plates following 3 hours of incubation more efficiently than the wild-type nisin (Rink et al., 2007).

Furthermore, it should be pointed out that bioengineering-based strategies have been employed for a variety of other applications; including upregulating production of

antimicrobial peptide (Cotter et al., 2006; Heinzmann et al., 2006), utilizing nisin biosynthesis machinery to secrete class II bacteriocins (Majchrzykiewicz et al., 2010), the post-translational modification of other bioactive peptides (Kluskens et al., 2009; Kuipers et al., 2004; Rink et al., 2005) and overcoming proteolytic resistance mechanisms (Field et al., 2019; Ottenwalder et al., 1995). The staphylococcal derived lantibiotics epidermin and gallidermin have also been the focus of mutational analysis, with the aim of generating derivatives with enhanced proteolytic resistance to trypsin (Ottenwalder et al., 1995). The creation of mutants that were resistant to proteases, while retaining bioactivity was more desirable. This study was partially successful in that staphylococcin mutations showed enhanced resistance to proteolytic enzymes, however the antimicrobial activity of the peptides was greatly reduced.

In addition to bioengineering approaches, the use of synthetic chemistry to create lantibiotic-like peptides is growing at pace, with many encouraging reports emerging (Arnusch et al., 2008; Cobb and Vederas, 2007; Ross et al., 2010). Chemical synthesis has enabled the production of potent nisin vancomycin hybrids and the chemical synthesis of the staphylococcal lantibiotic epilancin 15X (Arnusch et al., 2008; Knerr and Van Der Donk, 2012).

1.24 Bacterial defence systems against nisin

The main target of nisin is the cytoplasmic membrane where it binds to the lipid II and gets incorporated to form pores through which leads to the leakage of cellular contents resulting in destruction of the bacterial membrane (Prince et al., 2016). Over the years it has been postulated that nisin-resistant strains would emerge. Many Gram-positive bacteria have been shown to produce nisin-specific proteolytic enzymes, which cleave and inactivate the nisin molecule (Abee et al., 1995; Field et al., 2019).

Undoubtedly, the development of resistance mechanisms against nisin represents a main challenge in the development of novel food or clinical applications. These nisin-specific proteases are specific to nisin resistant strains and so far, have been confirmed to be produced by numerous bacterial species, including *Bacillus cereus*, *B. megaterium*, *B. polymyxa*, *L. lactis*, *S. aureus*, *S. capitis*, *Streptococcus agalactiae*, *S. thermophilus*, *Lactobacillus plantarum* and *E. faecalis*. Nisin resistance was reported to be provided by a specific nisin resistance gene (termed NSR), which is located on a 60-kb lactococcal plasmid, pNP40, and encodes a 35-kDa nisin resistance protein (NSR) (Field et al., 2019; Sun et al., 2009). A detailed investigation conducted by Sun and colleagues (Sun et al., 2009), demonstrated that NSR can render nisin inactivate by cleaving the peptide between positions 28 and 29 of the C-terminal end (between MeLan28 and Ser29) producing a truncated nisin molecule ($nisin^{1–28}$). Furthermore, the subsequent truncated $nisin^{1–28}$ peptide displays a significantly decreased efficiency in pore formation and a 100-fold decrease in bioactivity (Sun et al., 2009).

In addition to NSR proteases, another resistance mechanism has been identified in *S. agalactiae*. Khosa and colleagues (Khosa et al., 2013) identified an ABC transporter, termed NSR-FP. NSR-FP functions by exporting nisin into the external environment (Reiners et al., 2017). As previously mentioned, NSR captures and inactivates nisin by proteolytically removing the last six amino acid residues of the C-terminus, thus significantly reducing its bioactivity (Sun et al., 2009). However, when exposed to higher concentrations of nisin, it is unlikely that NSR would be capable of cleaving all the nisin molecules. Consequently, NSR-FP functions by providing a secondary resistance system to rescue the bacterial cell from nisin molecules that evade the first line of defence provided by NSR (Khosa et al., 2013; Reiners et al., 2017). The NSR-FP transport system

provides the bacterial strain with resistance up to 80 nM nisin, whereas a bacterial strain deprived of this transporter can only withstand a nisin concentration of 5 nM (Khosa et al., 2013). The NSR-FP system has been shown to recognize the nisin N-terminus. This was experimentally confirmed when the NSR-FP system provided resistance to lantibiotics nisin H (O'Connor et al., 2015) and gallidermin (Kellner et al., 1988), which share similarities with the N-terminus of nisin but have differences in the C-terminal region (Khosa et al., 2013).

It has been demonstrated that the level of lipid II in the bacterial membrane does not correlate with the development of nisin resistance. A study by Kramer and colleagues (Kramer et al., 2004) investigated whether variations in the levels of Lipid II were responsible for the differences in nisin sensitivity, using *M. flavus* and *L. monocytogenes* and their isogenic nisin-resistant variants. This study revealed that bacterial protoplasts containing varying amounts of lipid II in their cell membrane were equally sensitive to nisin (Kramer et al., 2004). Therefore, research groups have theorised that Gram-positive bacteria have acquired nisin resistance through the development of changes in the cell wall structure which is the most plausible explanation for acquirement nisin resistance (Draper et al., 2015).

Indeed, nisin has made a significant impact as a natural bio-preservative in the food industry for different types of foods, and also displays potent bioactivity against numerous food pathogenic bacteria, including *L. monocytogenes* and *S. aureus* (Field et al., 2015b, 2010b). However, the development of bacterial resistance mechanisms against nisin represents a major challenge for clinical use. Therefore, the need to explore alternative antimicrobial compounds that can overcome NSR mechanisms, remains paramount. Given the merits of bioengineering the nisin peptide, it may be possible to

create novel nisin derivatives with combined enhanced specific activity in addition to improved resistance to proteolytic cleavage by NSR.

1.25 Synergistic interaction between antimicrobials

From a food quality and safety perspective, the use of traditional bio-preservatives in food products such as chemical additives are being challenged, with consumers shifting towards natural and minimally processed foods. However, such demands increase the risks associated with foodborne pathogens, particularly *L. monocytogenes*. Accordingly, providing safe and high-quality food products, without antibiotic residue, and resistance development represents a major challenging for the global food industry.

While nisin has numerous applications in food preservation, resistance to nisin by *L. monocytogenes* has been reported, encouraging the search for alternative antimicrobials, either to replace or to be used in combination with nisin as a food preservative. A study conducted by Kaur and colleagues (Kaur et al., 2013) demonstrated that combinations of nisin, pediocin 34 and enterocin FH99 produce a more enhanced antimicrobial effect against *L. monocytogenes*, when compared to bacteriocins used alone. Similar results were reported by Hanlin and colleagues (Hanlin et al., 1993), that bacteriocin combinations would have greater antimicrobial effect to a sensitive population, since cells resistant to one bacteriocin might be killed by the other bacteriocin. Furthermore, Mulet-Powell and colleagues (Mulet-Powell et al., 1998) reported synergistic interactions between pairs of bacteriocins from LAB (nisin, pediocin AcH, lacticin 481, lactacin F, and lactacin B) when examined against 10 different indicator strains, including *Enterococcus faecalis, L. monocytogenes, L. innocua, L. ivanovii, L. welsheri, Lactobacillus helveticus, Lactobacillus fermentum, Pediococcus pentosaceus,* and *S. aureus*. The enhanced effectiveness of different bacteriocin combinations might be

explained by the fact that these bacteriocins belonging to different classes and vary greatly in the nature and sequence of amino acid residues. Similar results have been reported by Jamuna and colleagues (Jamuna et al., 2005) who demonstrated that the bacteriocins from *L. acidophilus* and *L. casei* (LABB and LABP, respectively) have greater antimicrobial activity in combination with nisin than when used alone against *L. monocytogenes* and *S. aureus* in liquid and food systems. Vignolo and colleagues (Vignolo et al., 2000) reported that the combined effect of lactocin 705, enterocin CRL35, and nisin against *L. monocytogenes* in meat slurry showed no viable counts following 3 hours of incubation. Jamuna and Jeevaratnam (2009) have also reported the synergistic effect of nisin and a bacteriocin from *Pediococcus acidilactici* to be more effective in inhibiting the growth of *L. monocytogenes* and *S. aureus* in sealed pouches of vegetable pulav.

Nisin has made a significant impact as a natural bio-preservative in the food industry and is one of only two natural preservatives to meet the standards of the European Union (EU) food additive list. As a result, the use of nisin is likely to increase in coming years. Although nisin displays potent bioactivity against foodborne pathogenic bacteria, including *L. monocytogenes* and *S. aureus* (Field et al., 2015b, 2010b; Jamuna and Jeevaratnam, 2009; Jamuna et al., 2005; Kaur et al., 2013; Vignolo et al., 2000), studies have reported the emergence of nisin resistant mutants of *L. monocytogenes* (Ming and Daeschel, 1993, 1995; Davies et al., 1996; Verheul et al., 1997). The development of bacteriocin resistance could have a major impact on their application as food bio-preservatives. A synergistic mixture of bacteriocins with different modes of action may enhance their antimicrobial effects and limit the risk of resistance development. In an effective synergistic interaction, nisin in combination with other bacteriocins would be used in reduced concentrations, which lowers the costs of using these compounds at an

industrial scale and makes the approach economically viable. Therefore, the need to explore bacteriocins combinations that reduce the risks of resistance development and display potent antimicrobial activity remains paramount.

Chapter 2

Identification and characterisation of capidermicin, a novel bacteriocin produced by *Staphylococcus capitis*

A manuscript based upon this chapter has been published in PLOS one.

Lynch D, O'Connor PM, Cotter PD, Hill C, Field D, Begley M. (2019) Identification and characterisation of capidermicin, a novel bacteriocin produced by *Staphylococcus capitis*. Tse H, editor. PLoS One. 2019 Oct 16;14(10):e0223541

2.1 Abstract

One hundred human-derived coagulase negative staphylococci (CoNS) were screened for antimicrobial activity using agar-based deferred antagonism assays with a range of indicator bacteria. Based on the findings of the screen and subsequent well assays with cell free supernatants and whole cell extracts, one strain, designated CIT060, was selected for further investigation. It was identified as *Staphylococcus capitis* and herein we describe the purification and characterisation of the novel bacteriocin that the strain produces. This bacteriocin which we have named capidermicin was extracted from the cell-free supernatant of *S. capitis* CIT060 and purified to homogeneity using reversed-phase high performance liquid chromatography (RP-HPLC). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric (MS) analysis revealed that the capidermicin peptide has a mass of 5,464 Da. Minimal inhibitory concentration (MIC) experiments showed that capidermicin was active in the micro-molar range against all the Gram-positive bacteria that were tested. Antimicrobial activity was retained over a range of pHs (2 – 11) and temperatures (10 – 121ºC x 15 mins). The draft genome sequence of *S. capitis* CIT060 was determined, and the genes predicted to be involved in the biosynthesis of capidermicin were identified. These genes included the predicted capidermicin precursor gene, and genes that are predicted to encode a membrane transporter, an immunity protein and a transcriptional regulator. Homology searches suggest that capidermicin is a novel member of the family of class II leaderless bacteriocins.

2.2 Introduction

The rise and spread of multidrug-resistant bacterial pathogens, coupled with a diminishing repertoire of effective antibiotics has necessitated the search for new alternative antimicrobial agents. Over the past decade, ribosomally synthesized natural peptides produced by a diverse group of bacterial species have received attention (Arnison et al., 2013)a. Compared to non-ribosomally synthesized antimicrobials, the ribosomally produced peptides are attractive for pharmaceutical applications as they could potentially be bioengineered to improve characteristics such as potency, stability, solubility etc. (Perez et al., 2014). One group of compounds, namely the bacteriocins, have attracted great interest due to their high potency (often active in the nano-molar range) and heat stability (Cotter et al., 2013).

It has been suggested that most bacteria produce at least one bacteriocin (Riley and Wertz, 2002). While the exact ecological function of bacteriocins is unknown, they may play a role in competition by directly killing competing bacteria, function as colonizing peptides or function as signalling molecules to communicate with other bacteria or the host (Dobson et al., 2012). Bacteriocin-producing bacteria have been isolated from a wide variety of sources including food (Kelly et al., 1996), soil (Yanagida et al., 2006) and the intestines of fish and animals (Collins et al., 2016; Ringø et al., 2018). In addition, several members of the human microbiota have been shown to produce bacteriocins. For example we have previously employed both functional and *in silico* approaches in our laboratory to identify novel bacteriocins from the human gut microbiome (Begley et al., 2009; Collins et al., 2017; Lakshminarayanan et al., 2013; O'Connor et al., 2015; O'Sullivan et al., 2011; Walsh et al., 2015). Skin-derived bacteria have also been shown to produce, or

shown potential to produce, bacteriocins (Cameron et al., 2015; Cogen et al., 2008; Janek et al., 2016; Kumar et al., 2017; O'Sullivan et al., 2019; Zipperer et al., 2016).

For the current study, we have focused on examining the antimicrobial ability of coagulase negative staphylococci (CoNS) that were isolated from human skin or human blood with the aim of identifying novel bacteriocin-producers. CoNS are considered part of the normal commensal bacteria of the skin and are thought to act as host guardians by targeting pathogens (Christensen and Bruggemann, 2014). We postulate that bacteriocin production plays an important role in this function and in support of this hypothesis Nakatsuji and colleagues (Teruaki Nakatsuji et al., 2017) have shown that antimicrobial-producing CoNS are deficient in subjects with atopic dermatitis and reintroduction of these strains decreased colonization with *Staphylococcus aureus*. While numerous lantibiotics have been identified from CoNS, including epidermin from *Staphylococcus epidermidis*, gallidermin from *Staphylococcus gallinarium* and hominicin from *Staphylococcus hominis* (Götz et al., 2014), analysis of the entire genome sequences of publicly available CoNS genomes suggests that there are potentially many novel CoNS-derived bacteriocins that have not yet been functionally characterized (M. Begley; unpublished data).

The aim of this study was to screen one hundred human-derived CoNS for antimicrobial activity using agar-based deferred antagonism assays with a range of indicator bacteria. One CoNS strain (CIT060) was selected for further investigation and it was identified as *Staphylococcus capitis* and herein we describe the purification and characterisation of the novel bacteriocin that the strain produces.

2.3 Materials and Methods

2.3.1 Bacterial strains and growth conditions

The bacterial indicator strains used in this study are listed in Table 2. Strains were grown in either Brain Heart Infusion (BHI) broth at 37ºC or in M17 broth supplemented with 0.5% glucose (GM17) at 30ºC as indicated in the Table. All media was purchased from Oxoid and prepared according to the manufacturer's recommendations.

2.3.2 Assembly of a bank of coagulase negative Staphylococci (CoNS)

The 100 CoNS strains were originally isolated from human skin swabs (obtained by swabbing the retro auricular crease i.e. behind the ear, the alar crease i.e. the side of the nose or the wrist), or from human blood samples at Cork University Hospital (CUH). Aliquots from archived stocks that are stored at -80ºC at Munster Technological University (MTU) were plated onto Mannitol Salt Agar (MSA) and incubated at 37ºC for 16-18 hours. Single colonies were selected from the MSA plates and re-streaked onto BHI agar for purity determination. Strains were identified using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) at CUH. Briefly, single fresh colonies (from BHI agar plates incubated at 37ºC for 16-18 hours) were directly applied to the MALDI-TOF stainless steel target plate. After application, each bacterial colony was covered with 0.8 µL of matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid [HCAA] in 50 % acetonitrile-2.5 % trifluoroacetic acid) (Bruker Daltonik, GmbH, Germany). The data collected was classified in accordance to Bruker Taxonomy database of CUH.

The 100 strains were re-stocked in two separate master 96 well plates (50 in each plate). Overnight cultures of the 100 strains were prepared by selecting a single pure colony from BHI agar and adding it to 10ml of BHI broth and incubating at 37ºC for 16-20 hours.

After incubation, 100µl of each fresh overnight culture was added to a specific well in the 96 well plate, and 100µl of sterile 80% glycerol was added to each well. The master stock 96 well plates were stored at -80ºC. Prior to use, the plates were thawed at room temperature.

2.3.3 Screening of a bank of CoNS for antimicrobial activity

Agar-based deferred antagonism assays were carried out with a selection of indicator bacteria (listed in Table 2). The 100 CoNS strains were replicated onto BHI agar from the 96 well stock plates using a 96-pin replicator (Boekel). Plates were incubated at 37ºC for 16 – 18 hours after which the surface of the agar plate was subjected to UV treatment for 30 minutes (High performance UV transmitter, Upland, Ca, USA). 30µL of fresh overnight cultures of indicator strains were added to 20ml of relevant sloppy/soft agar (BHI/GM17 broth supplemented with 0.75% w/v agar) and poured over the replicated plates. Plates were incubated at 37ºC for 12 – 16 hours after which they were examined for zones of inhibition.

2.3.4 Investigation of the antimicrobial activity of cell-free supernatants and crude whole cell extracts

Short-listed CoNS were grown overnight in BHI broth at 37ºC under vigorous continued shaking (130 rpm), and a 1% inoculum of the cultures were added to 50 ml clarified BHI broth (prepared by passing BHI broth through XAD-16N beads (Sigma-Aldrich) prior to autoclaving) and incubated with vigorous continued shaking at 37ºC. Following incubation, 50 ml of bacterial cells were centrifuged at 7,000 rpm for 20 minutes, supernatant removed and retained, i.e., cell-free supernatant (CFS). The cell pellets were resuspended in 7 ml 70% isopropanol (IPA) 0.1% trifluoroacetic acid (TFA) and stirred vigorously for 3 hours. Cell debris was removed through centrifugation and the

supernatants were retained and referred to as whole cell extracts (WCE). CFS and WCE were examined for antimicrobial activity in an agar well diffusion assay using *L. lactis* HP as the indicator strain. 50ml of molten agar was seeded with 100 µL of *L. lactis* HP that was grown overnight at 30°C, and once solidified, 4.6mm holes were bored with a sterile glass pipette. 50 µL of CFS and WCE were added to separate wells. Plates were incubated at 30°C for 16-18 hours after which they were examined for zones of inhibition.

2.3.5 Purification of capidermicin from *S. capitis* CIT060

Capidermicin was purified from *S. capitis* CIT060 using a method described by Field and colleagues (Field et al., 2015c) with modifications. Three litres of clarified BHI (cBHI) broth was inoculated (1%) with *S. capitis* CIT060 and incubated for 18-20 hours at 37°C under vigorous continued shaking (130 rpm). The culture was centrifuged at 7,000 rpm for 15 minutes. The cell pellet was removed, and the supernatant was retained and passed through 60g of Amberlite XAD16N beads (Sigma Aldrich). The beads were washed with 30% ethanol, and the peptide was eluted in 500ml 70% isopropanol (IPA) containing 0.1% trifluoroacetic acid (TFA). The IPA was evaporated using a rotary evaporator (Buchi) and the sample pH adjusted to 4 before applying to a 60 ml Strata C-18 E column (Phenomenex) that was previously pre-equilibrated with 60 ml methanol (Fisher Scientific, UK) and 60 ml H_2O. 100 ml of 30% ethanol was used to wash the column and the peptide was eluted in 60 ml of 70% IPA, 0.1% TFA. 10 ml aliquots were concentrated to 2 ml through the removal of IPA by rotary evaporation. 2.0 ml aliquots were applied to a Phenomenex (Phenomenex, Cheshire, UK) C12 reverse phase (RP)-HPLC column (Jupiter 4u proteo 90 Å, 250 × 10.0 mm, 4 µm) previously equilibrated with 25 % acetonitrile containing 0.1% TFA. The column was subsequently developed in a gradient of 25 % acetonitrile containing 0.1 % TFA to 50 % acetonitrile containing 0.1 % TFA

from 10 to 45 minutes at a flow rate of 2ml min^{-1}. The relevant active fractions were collected and pooled, subjected to rotary-evaporation to remove the acetonitrile and freeze-dried. The purity of the peptide was analysed by MALDI-TOF Mass Spectrometry (Cotter et al., 2006).

2.3.6 Minimum inhibitory concentration (MIC) assays

MIC determinations were carried out in triplicate in 96 well microtitre plates pre-treated with bovine serum albumin (BSA) as described by (Field et al., 2012). 200µL of 1X phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (PBS/BSA) was added to each well and incubated for 30 minutes at 37^{o}C. The wells were washed with 200µL PBS and allowed to dry. Target strains were grown overnight in the appropriate medium and temperature conditions, sub-cultured into fresh broth and allowed to grow to an OD_{600} of approximately 0.5, and diluted to a final concentration of 10^5 CFU/ml in a volume of 200µL broth. Lyophilised capidermicin was resuspended in cation adjusted BHI broth to a desired concentration, and a 2 - fold dilution of the peptide was made in the 96 well plate. The target strain was then added and after incubation at 30^{o}C or 37^{o}C for 16 hours the MIC was read as the lowest peptide concentration causing inhibition of visible growth.

2.3.7 Stability assays with capidermicin

The susceptibility of purified capidermicin peptide to temperature, pH and protease enzymes was investigated through well diffusion assays. To determine temperature stability the purified peptide was subjected to 10, 30, 40, 50, 80, 90, 121^{o}C for 15 minutes. Bioactivity was then determined by carrying out an agar well diffusion assay with *L. lactis* HP as the bacterial indicator. To evaluate the susceptibility of the peptide to varying pH values, the purified peptide solution was adjusted to pH 2 – 11 using 1M HCl or 1M

NaOH, respectively. After a brief vortex, the peptide was incubated at room temperature for 15 minutes, and the bioactivity was again determined with the well diffusion assay with *L. lactis* HP. The susceptibility of the peptide to proteolytic cleavage was analysed using trypsin, α-chymotrypsin, pepsin and proteinase K (Sigma-Aldrich). Protease enzymes were dissolved in 100 mM Tris-HCl – 10 mM $CaCl_2$, to a final concentration of 100 µg/ml. Preparations of capidermicin were incubated with the various enzymes at 37^{o}C for 4 hours and the bioactivity of capidermicin was reassessed using the well diffusion assay described above.

2.3.8 Sequencing of *S. capitis* CIT060 genome

DNA extracted from *S. capitis* CIT060 was quantified using a Qubit high sensitivity assay (Invitrogen), and diluted to 0.2ng/µl. Genomic libraries were then prepared using the Nextera XT Library preparation kit (Illumina) essentially as described in the manufacturer's protocol with the following exceptions. Firstly, the tagmentation time was extended to 7min. Following addition of indices, products were cleaned using AMPure XP magnetic bead-based purification, as described in the manufacturer's protocol and then secondly, in place of the bead based normalisation, the products were run on an Agilent Bioanalyser to determine average fragment size (Agilent) and quantified again by Qubit. Cleaned genomic fragments were then pooled equimolarly. The sample pool (4nM) was denatured with 0.2N NaOH, then diluted to 6pM and combined with 10% (v/v) denatured 6pM PhiX, prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform in the Teagasc sequencing facility, Moorepark, Fermoy, using a 2 x 300 cycle V3 kit, following standard Illumina sequencing protocols.

2.3.9 *In silico* analyses of the predicted capidermicin gene cluster and encoded proteins

Following sequencing, the reads were assembled using Spades v. 3.5.0 (Bankevich et al., 2012). Opening reading frames (ORFs) were predicted using Prodigal V.1.20 (Hyatt et al., 2010) and assigned a putative function based on BLASTp analysis at NCBI (http://www.ncbi.nlm.nih.gov/) and Pfam matches (EMBL-EBI) (Finn et al., 2016). Any genomic regions which were identified as potentially containing antimicrobial-encoding genes were visualised using Snapgene Viewer (GSL Biotech; available at www.snapgene.com). These regions were manually annotated, and BLAST searches were performed with ORFs. Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2)) (Kelley et al., 2015) was used to generate a putative three-dimensional structure of the capidermicin peptide using two homologous peptides as templates - aureocin A53 (accession number AAN71834.1) and lacticin Q (accession number BAM66973.1). The sequence alignment of peptides of interest was performed using CLUSTAL OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.5 Results

2.4.1 Screening a bank of CoNS strains for antibacterial activity

A bank of 100 CoNS strains was assembled. All strains were phenotypically characterised using MSA agar plates and identified by MALDI-TOF analysis. The bank consisted of various staphylococcal species including 83 *S. epidermidis*, 7 *S. capitis*, 3 *S. haemolyticus*, 3 *S. hominis*, 2 *S. warneri*, 1 *S. saprophyticus* and 1 *S. simulans*. The 100 strains were examined using agar-based deferred antagonism assays for their ability to inhibit a selection of indicator strains (24 in total), including enterococci, lactococci, *Micrococcus*, streptococci and other *Staphylococcus* strains. Zones of inhibition were observed for 94 strains; representative images are shown in Figure. 8. Six of the *S. epidermidis* strains did not demonstrate antimicrobial activity under the conditions tested. 15 of the 94 strains displayed a broad spectrum of activity, inhibiting 10 or more indicator strains. One strain, namely S. *capitis* CIT060, was capable of inhibiting 14 of the 24 bacterial indicators (*B. cereus* DPC6089, *E. faecailis* MR103, *G. kaustophilius* DSM7263, *L. lactis* subsp *cremonis* IP5, *L. lactis* HP, *M. luteus* DSM1790, *S. aureus* NCDO1499, DPC5297, Newman, and RF122, *S. lugdunensis*, *S. pseudintermedius DSM21284*, *S. intermedius* DSM 20373 and *S. dysgalactiae* ATCC43078).

2.4.2 Purification of capidermicin from *S. capitis* CIT060

Initial experiments with cell-free supernatant and crude whole cell extracts prepared from *S. capitis* CIT060 suggested that the antimicrobial produced by the strain is primarily in the supernatant (data not shown). Consequently, efforts focused on purifying an antimicrobial peptide from culture supernatants as described in the Methods section. MALDI-TOF MS analysis revealed that the purified peptide, that we named capidermicin, had a mass of 5,464 Da (Figure. 9).

The antimicrobial activity of purified capidermicin, as determined by agar well diffusion assays with *L. lactis* HP, remained unaffected following heat treatment at 10, 30, 40, 50, 80, 90 or 121^{o}C for 15 minutes. The peptide was also able to maintain full bioactivity following exposure to pH 2 – 8. A 70% decrease in zone of inhibition observed at pH 9 – 11. Exposing capidermicin to α-chymotrypsin and pepsin had no effect on the bioactivity of the peptide. However, treatment with trypsin or proteinase K resulted in a 50% reduction in activity. The specific activity of capidermicin was assessed using standard MIC broth-based assay and results are presented in Table 3. It was observed that capidermicin was active against the selected target bacteria in the nano- and micromolar range.

2.4.3 *In silico* analyses of the predicted capidermicin gene cluster and encoded proteins

The draft genome of *S. capitis* CIT060 was analysed using a variety of *in silico* tools for the presence of potential antimicrobial peptide encoding genes. Three areas of interest were identified which included a lantibiotic gene cluster, a phenol soluble modulin (PSM) gene cluster and an aureocin-like gene cluster.

The first area of interest contains a gene that is predicted to encode a 44 amino acid peptide that is homologous to the lantibiotic gallidermin of S*taphylococcus gallinarum* (accession number U61158.1). The 44 amino acid peptide is predicted to encode a peptide with a mass of 5 kDa. However, a corresponding mass could not be detected from our bioactive HPLC fractions or purified capidermicin preparations. The second area of interest contains four genes that are predicted to encode PSMβ peptides. Phenol-soluble modulins (PSMs) are a recently discovered group of amphipathic peptides that have multiple roles in staphylococcal pathogenesis. They have been shown to exhibit antimicrobial activity (Kumar et al., 2017). All four predicted peptides contain the

conserved domain of staph_haemo superfamilies (pfam05480), their amino acid sequences are identical to the PSMβ peptides previously reported in the literature (Cameron et al., 2015; Kumar et al., 2017), and they are predicted to have masses of 4.57 kDa, 4.54 kDa, 4.62 kDa and 4.79 kDa. Again, corresponding masses could not be detected from our bioactive HPLC fractions or purified capidermicin preparations. The genetic organization of the final area of interest is shown in Figure 10 and the predicted functions of the putative gene products are shown in Table 4. *Orf4* was predicted to encode a peptide that is homologous to a number of previously characterised bacteriocins including lacticin Z produced by *Lactococcus lactis* QU14 (46% identity; accession number BAF75975), aureocin A53 produced by *Corynebacterium jeikeium* (41% identity; accession number WP010976360) and an aureocin-like bacteriocin produced by *Lactococcus ruminis* (57% identity; accession number SEM89646). More distant homologues include BacSp222 (32% identity; accession number A0A0P0C3P7) and Lactolisterin BU (44% identity; accession number SDR48784). A Clustal Omega alignment was carried out to compare the predicted peptide encoded by *orf4* and previously characterised bacteriocins, and the relatedness of the peptides is depicted in Figure 11.

The mass of the putative *orf4*-encoded peptide was predicted to be 5,438 Da by *in silico* tools (under Genbank accession MN234131). However, as the homologue aureocin A53 contains an N-formylated methionine (Netz et al., 2002c)), and the start codon of *orf4* was noted to be TTG (Figure 10), a revised theoretical mass of 5,466 Da was predicted. This mass is virtually identical to the mass of the capidermicin peptide that we purified from *S. capitis* CIT060 (Figure 9).

Capidermicin is predicted to be cationic with a putative net charge of 5.34 (theoretical pI = 10.22) and is rich in lysine residues (14%). A 3D structural model is presented in Figure 12. The peptide is predicted to be composed of four α-helices.

2.5 Discussion

The aim of the current study was to screen 100 human-derived CoNS for antimicrobial activity. Agar-based deferred antagonism assays revealed that 94 of the strains reproducibly inhibited at least some of the tested indicator bacteria. Six strains did not show antimicrobial activity under the test conditions employed but it is possible that these strains may demonstrate activity under other conditions, such as different growth medium or different indicator bacteria. Janek and colleagues (Janek et al., 2016) carried out a similar agar-based screen to determine the frequency of antimicrobial production by 89 human nasal *Staphylococcus* isolates. 77 of the 89 isolates (86.5%) exhibited antimicrobial activity. When taken together, the findings of both studies suggest that antimicrobial production is a common phenotype among CoNS isolates. In contrast, a recent study by O' Sullivan and colleagues (O'Sullivan et al., 2019), which describes a similar screen with human skin-derived staphylococci reports that only 101 possible antimicrobial-producers were identified from over 90,000 colonies that were screened for antimicrobial activity. The difference in the frequency of isolation of antimicrobial producers between that study (0.112%), the study by Janek *et al*. (86.5%) and our study (94%) may be because O' Sullivan *et al*. used only one indicator organism in their screen (*Lactobacillus delbrueckii* subsp. *bulgaricus*) while a variety of indicators were used in the other two studies. It is possible that antimicrobials produced by CoNS may not inhibit *L. delbruecki* or activity may be too low to be detected in deferred antagonism assays.

Interestingly, functional screens of human intestine-derived bacteria report isolation of antimicrobial producers at a low frequency. Lakshminarayanan and colleagues (Lakshminarayanan et al., 2013) screened over 70,000 faecal bacteria for their ability to inhibit the indicators *Lactobacillus bulgaricus* LMG6901 and *Listeria innocua*

DPC3572 and only identified 55 antimicrobial producers (a frequency of 0.08%). The *in silico*-based investigations of Zheng and colleagues (Zheng et al., 2015) revealed that the human gut microbiome had the lowest frequency of putative bacteriocin genes of all the human body sites investigated. It is possible that production of antimicrobials by CoNS will increase their fitness in order to compete and survive on human skin. The limited availability of nutrients and water in this environment compared to the gastrointestinal tract may mean that the skin is a more competitive environment hence explaining the higher frequency of antimicrobial producers in screens with skin-derived bacteria compared to gut-derived bacteria. As humans and their microbes have co-evolved it is likely that production of antimicrobials by CoNS plays a beneficial role for the host, perhaps by contributing to the role of the skin as the body's first line of defence by protecting against pathogenic bacteria. It is also possible that the antimicrobials may act as signalling molecules and interact with the human immune system (Dobson et al., 2012).

The 94 CoNS that demonstrated antimicrobial activity in the initial screen were shortlisted to 15 based on their inhibition spectra and the activity of cell-free supernatants and whole cell extracts in well assays. *S. capitis* CIT060 was selected for further investigation as it demonstrated a broad inhibition spectrum and the results of well assays suggested that the antimicrobial could potentially be purified using methods that we currently use for bacteriocins in our lab (Field et al., 2015c) . We also noted that there are very few reports of functionally characterized *S. capitis* antimicrobials in the literature. While antimicrobial production by *S. capitis* strains has been shown by agar-based studies (Janek et al., 2016; O'Sullivan et al., 2019) and antimicrobial genes have been identified in *S. capitis* genomes by *in silico* based methods (Cameron et al., 2015; Carson et al.,

2017), to our knowledge the only two functional characterization studies in the literature are those of Sugai and colleagues (Sugai et al., 1997a), who reported the purification of the glycylglycine endopeptidase ALE-1 from *S. capitis* EPK1 that is similar to the bacteriocin lysostaphin, and Kumar and co-workers (Kumar et al., 2017) who chemically synthesized and characterized phenol soluble modulins.

A 5,464 Da peptide, that we named capidermicin, was purified from *S. capitis* CIT060 cell free supernatants. The antimicrobial activity of the peptide was confirmed, and it was shown to retain its activity over a range of pH and temperatures. Analysis of the *S. capitis* CIT060 genome revealed the presence of 6 potential antimicrobial encoding genes (2 bacteriocins, 4 PSMs) but based on mass we deduced that the antimicrobial peptide that we purified was the product of the aureocin-like gene cluster. A gene encoding a potential 44 amino acid lantibiotic similar to gallidermin was also identified in the genome of *S. capitis* CIT060. Carson and colleagues (Carson et al., 2017) reported the presence of lanthipeptide gene clusters in the genomes of the 12 *S. capitis* isolates analysed (*S. capitis* 1319, 3379, 3769, 4275, 6079, 807, 1187, 1642, 2477, 2643, 4830 and 5871). Three of these strains were also shown to contain a sactipeptide gene cluster (*S. capitis* 1319, 2487 and 3379)(Carson et al., 2017)). Kumar and colleagues (Kumar et al., 2017) identified four distinct gene clusters with the ability to encode antimicrobial peptides (epidermicin, gallidermin and phenol-soluble modulins) in *S. capitis* TE8. While the lantibiotic did not seem to be produced by *S. capitis* CIT060 under the experimental conditions used, the production of more than one bacteriocin by a bacterium is not uncommon. Lactococci commonly produce more than one bacteriocin and *Lactococcus lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 strain produces at least two bacteriocins (Kojic et al., 2006; Miljkovic et al., 2016). Similarly, *Staphylococcus aureus* 4185, a

bovine mastitis isolate, was shown to produce five antimicrobial peptides (named peptides A – E) (Ceotto et al., 2010a).

In silico analyses suggest that capidermicin is a novel member of the class II leaderless bacteriocin family. It shows most similarity to members of the aureocin 53-like sub-group of the family which includes aureocin A53 produced by *S. aureus* A53 (Netz et al., 2002c), lacticin Z produced by *L. lactis* QU14 (Iwatani et al., 2007b), lacticin Q produced by *L. lactis* QU5 (Fujita et al., 2007) and epidermicin NI01 produced by *S. epidermidis* strain 224 (Sandiford and Upton, 2012). All of the bacteriocins within the group are 34-53 amino acid peptides, are highly cationic and are characterised by their lack of an N-terminal leader sequence during biosynthesis meaning that they do not undergo any post-translational modifications and become active shortly after translation (Perez et al., 2018). While the genes encoding the immunity and secretion machinery have been experimentally determined for lacticin Q and Z (Iwatani et al., 2013, 2012) , aureocin A53 (Nascimento et al., 2012) and aureocin A70 (Coelho et al., 2014; Netz et al., 2001);, compared to other the classes of bacteriocins there is little known about the biosynthesis of leaderless bacteriocins and they have been referred to as the most enigmatic and poorly understood group of bacteriocins (Perez et al., 2018). Similar to lacticin Q (48%) and aureocin A53 (Acedo et al., 2016), capidermicin is predicted to be α-helical globular molecule.

MIC assays revealed that capidermicin was active against Gram-positive bacteria at low concentrations (μM/nM). Similar findings have been reported for epidermicin NI01 (Sandiford and Upton, 2012) and lacticin Q (Fujita et al., 2007). While capidermicin is insensitive to α-chymotrypsin and pepsin, a 70% reduction in activity was observed when it was treated with proteinase K or trypsin. A similar decrease in activity has

previously been observed for epidermicin NI01, which displayed a 75% and 50% reduction in activity for proteinase K and trypsin, respectively (Sandiford and Upton, 2012). Resistance to proteases has been reported for other staphylococcal bacteriocins, including aureocin A53 and BacCH91 (Netz et al., 2002a; Wladyka et al., 2013). Capidermicin showed high stability under acidic, alkaline and neutral conditions, which has also been reported for aureocin A53 (Netz et al., 2002c), lacticin Z (Iwatani et al., 2007b) and lacticin Q (Fujita et al., 2007). It has previously been noted that the high stability of leaderless bacteriocins together with the simplicity of their biosynthesis may make them more attractive from a commercial view point compared to other bacteriocins (Perez et al., 2018).

A 3D structural model is presented in Figure 12. The peptide is predicted to be composed of four α-helices, and exhibits a recurring three dimensional structural motif found among many linear leaderless bacteriocins (lacticin Q, aureocin A53) and similar to that found in a larger superfamily of proteins known as saposin-like peptides (Towle and Vederas, 2017). All the helices of capidermicin are amphipathic whereby hydrophobic residues are oriented inward that pack to give a hydrophobic core and the hydrophilic residues are exposed on the surface in the same manner as LnqQ (data not shown). Similarly, both peptides have highly cationic surfaces. Capidermicin has 7 lysine residues which are well distributed throughout its primary structure. Indeed, all lysine residues are situated on the surface and are in fact found in the exact same locations to LnqQ (in capidermicin K3, K10, K14, K23, K44 and K50). Notably, although LnqQ and the closely related aureocin A53 are composed of four distinct α-helical structures that are structurally identical to each other, both have varying antimicrobial activity spectrums against Gram-positive bacteria (Acedo et al., 2016). Studies have shown that LnqQ

permeates target membranes by forming toroidal pores (4.6–6.6 nm in diameter), which facilitate the leakage of cellular contents (Yoneyama et al., 2009). However, a more recent study demonstrated that LnqQ was able to induce cell death even without the formation of pores (Li et al., 2013). AucA was also proposed to permeabilize cell membranes causing the leakage of essential molecules, dissipation of membrane potential, and cessation of macromolecular synthesis but without the formation of discrete pores (Netz et al., 2002a). Given the structural similarity of capidermicin to LnqQ and A53, it is likely that capidermicin can also permeabilize cell membranes resulting in cell death.

In conclusion, we report that our screening experiment revealed a large frequency of antimicrobial production by human CoNS isolates and we describe the subsequent identification and characterization of a novel bacteriocin from *S. capitis*. Future work in our laboratory will include the chemical synthesis of the capidermicin peptide for additional *in vitro* experiments to confirm activity and antimicrobial spectrum, similar to studies conducted on other leaderless bacteriocins including garvicin KS and epidermicin NI01 (Ovchinnikov et al., 2016; Sandiford and Upton, 2012). In the case of epidermicin NI01, chemical synthesis permitted the generation of sufficient peptide for further analyses including *in vivo* studies (Gibreel and Upton, 2013; Halliwell et al., 2017). Moreover, accessibility to capidermicin by chemical synthesis would provide a means for peptide engineering investigations to be carried out i.e. molecular engineering to enhance the potency, improve pharmacological properties, increase peptide stability and potentially modify the spectrum of activity. It may also provide more detail regarding the importance of the formylated methionine at the N-terminus for the antimicrobial activity of capidermicin. Future experiments will also include mutational analysis of all of the genes that are predicted to be involved in capidermicin production. A comprehensive gene

disruption of the capidermicin biosynthetic cluster will be carried out, similar to that of Iwatani and colleagues (Iwatani et al., 2013) whereby each gene of the lacticin Q operon (lnqQBCDEF) was individually deleted and the impact on production and immunity evaluated.

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Table 2: Bacterial strains used in this study.

Bacterial strains	Culture medium and temperature	Source
100 CoNS isolates	BHI at 37°C	MTU Culture Collection
Staphylococcus epidermidis TU3298 Positive control (epidermin producer), used during antimicrobial screening	BHI at 37°C	Teagasc, Culture Collection
Staphylococcus capitis CIT060	BHI at 37°C	MTU Culture Collection
Indicator Strains		
Bacillus cereus DPC 6089	BHI at 37°C	UCC culture Collection
Enterococcus faecailis MR103	BHI at 37°C	UCC culture Collection
Geobacillus kaustophilus DSM 7263	BHI at 55°C	UCC culture Collection
Geobacillus stearothermophilus ATCC 12930	BHI at 55°C	UCC culture Collection
Lactococcus cremoris IP5	GM17 at 30°C	UCC culture Collection
Lactococcus lactis HP	GM17 at 30°C	UCC culture Collection
Lactococcus lactis MG1363	GM17 at 30°C	UCC culture Collection
Lactococcus lactis NZ9800	GM17 at 30°C	UCC culture Collection
Micrococcus luteus DSM1790	BHI at 37°C	UCC culture Collection
Staphylococcus aureus 5971	BHI at 37°C	UCC culture Collection
Staphylococcus aureus DPC 5243	BHI at 37°C	UCC culture Collection
Staphylococcus aureus DPC5297	BHI at 37°C	UCC culture Collection

Bacterial strains	Culture medium and temperature	Source
Staphylococcus aureus NCDO 1499	BHI at 37^{o}C	UCC culture Collection
Staphylococcus aureus Newman	BHI at 37^{o}C	UCC culture Collection
Staphylococcus aureus RF122	BHI at 37^{o}C	UCC culture Collection
Staphylococcus epidermidis (UCC strain)	BHI at 37^{o}C	UCC culture Collection
Staphylococcus gallinarium 4616	BHI at 37^{o}C	UCC culture Collection
Staphylococcus intermedius DSM 20373	BHI at 37^{o}C	UCC culture Collection
Staphylococcus lugdunesis	BHI at 37^{o}C	UCC culture Collection
Staphylococcus pseudintermedius DSM 21284	BHI at 37^{o}C	UCC culture Collection
Streptococcus agalactiae ATCC 13813	BHI at 37^{o}C	UCC culture Collection
Streptococcus dysgalactiae ATCC43078	BHI at 37^{o}C	UCC culture Collection
Streptococcus pneumoniae (UCC strain)	BHI at 37^{o}C	UCC culture Collection
Streptococcus pyogenes NCDO 2381	BHI at 37^{o}C	UCC culture Collection

BHI = Brain heart infusion; GM17 = M17 broth + 0.5% glucose; MTU = Munster Technological University; UCC = University College Cork.

Table 3: Minimum inhibitory concentration (MIC) values of purified capidermicin against a range of Gram positive indicators. Identical MICs values were obtained in three independent determinations.

Species and Strain	MIC (µg/ml)	MIC (nM/µM)
Lactococcus lactis HP	19 µg/ml	3.4 µM
Listeria Monocytogenes EGDe	3.75 µg/ml	0.7 µM
Staphylococcus aureus NCDO 1499	3.1 µg/ml	0.6 µM
Staphylococcus aureus SA113	10 µg/ml	1.8 µM
Staphylococcus intermedius DSM20373	40 µg/ml	7.3 µM
Staphylococcus pseudintermedius DSM21284	10 µg/ml	1.8 µM
Staphylococcus pseudintermedius DK729	10 µg/ml	1.8 µM
Micrococcus luteus DSM1790	100µg/ml	18 µM

Table 4: *In silico* analysis of the genes predicted to be involved in the biosynthesis of capidermicin.

ORF	Locus tag	Gene Size (bp)	Closest homolog of predicted encoded protein	Protein sequence identity to closest homolog (Genbank Acession number)	Predicted Function
ORF 1	60_02323	738	Plasmid replication initiator protein of *Listeria monocytogenes*	98% (WP_096929472.1)	Plasmid replication
ORF 2	60_02322	1407	YdbT-like protein of *S. aureus*	29% (WP_032072953.1)	Self-immunity
ORF 3	60_02321	405	YdbS-like protein of *S. intermedius*	38% (COG3402)	Self-immunity
ORF 4	60_02320	153	Aureocin A53 – like protein	46% (AF447813)	Structural gene
ORF 5	60_02319	558	Membrane protein of *Staphylococcus* sp.TE8	76% WP_082243241.1)	Unknown
ORF 6	60_02318	243	Hypothetical protein of *S. epidermidis*	95% (WP_049397332.1)	Unknown
ORF 7	60_02317	624	Transposase of *Staphylococcus*	100% (WP_017176851.1)	Transposon
ORF 8	60_02316	516	Hypothetical protein	99% (WP_020368224.1)	Unknown
ORF 9	60_02315	996	Putative sulfate exporter family transporter	100% (WP_070441690.1)	Transport of peptide
ORF 10	60_02314	822	Transcriptional Regulator	100% (WP_070441693.1)	Gene Regulation

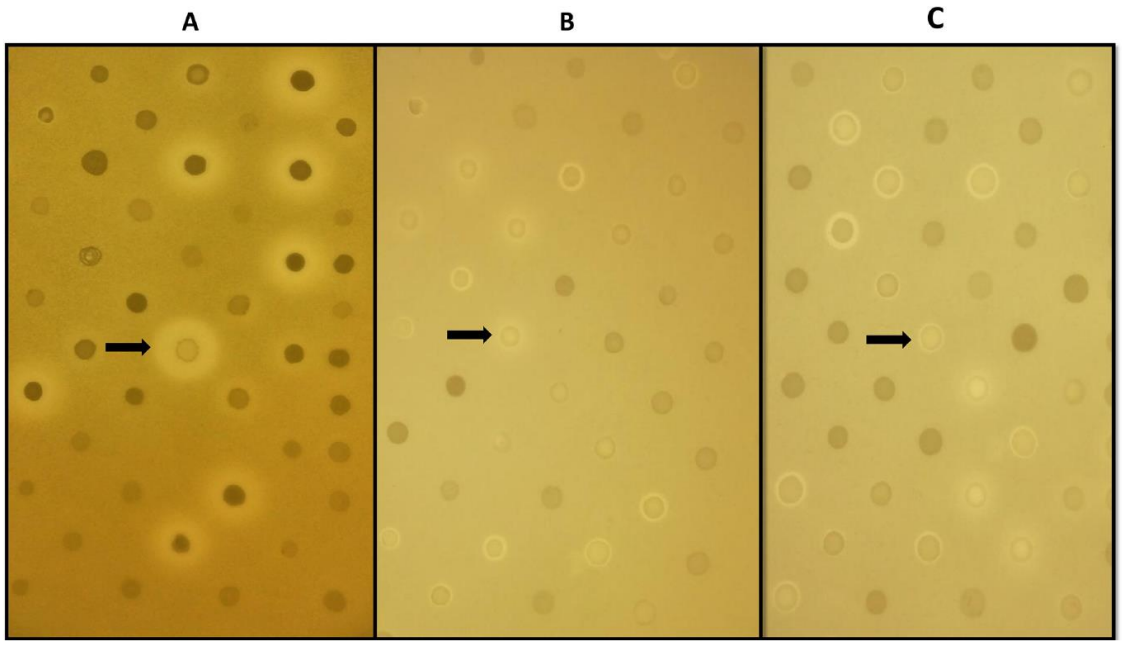

Figure 8. Representative images of the results obtained during the screen of 100 CoNS strains for antimicrobial activity. CoNS were replicated from master stock 96 well plates onto BHI agar using a 96-pin replicator. Plates were incubated at 37ºC overnight after which they were overlaid with sloppy agar containing relevant indicator bacteria. For the plates shown the indicators used were (A) *M. luteus* DSM1790, (B) *S. aureus* NCDO1499 and (C) *S. pseudintermedius* DSM21284. The arrows indicate the position of *S. capitis* CIT060 on the plates.

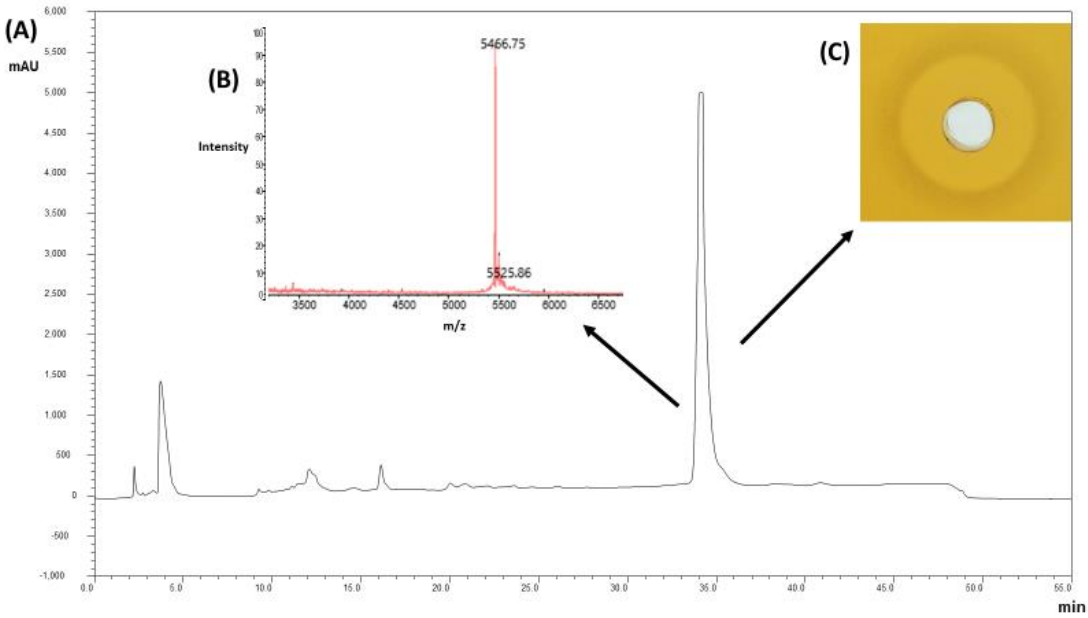

Figure 9. **(A)** Reversed – phase high performance liquid chromatography (RP-HPLC) profile for the purification of capidermicin using a Phenomenex C12 reverse-phase column, at a flow rate of 2ml/min. **(B)** MALDI-TOF Mass Spectrometry of lyophilized capidermicin revealed a mass of 5,464 Da. MALDI TOF MS chromatogram above indicates the presence of capidermicin (5464.39) and the K+ adduct ion (5502.61) and the doubly charged ion (2732.69) (2732 x 2 =5464). **(C)** Antimicrobial activity of HPLC bioactive fractions was determined using well diffusion assay using *L. lactis* HP as the indicator strain. GM17 agar was seeded with *L. lactis* HP, wells were bored, 50µL of the HPLC fraction was added to the well, and plates were incubated at 30^{o}C for 16 hours.

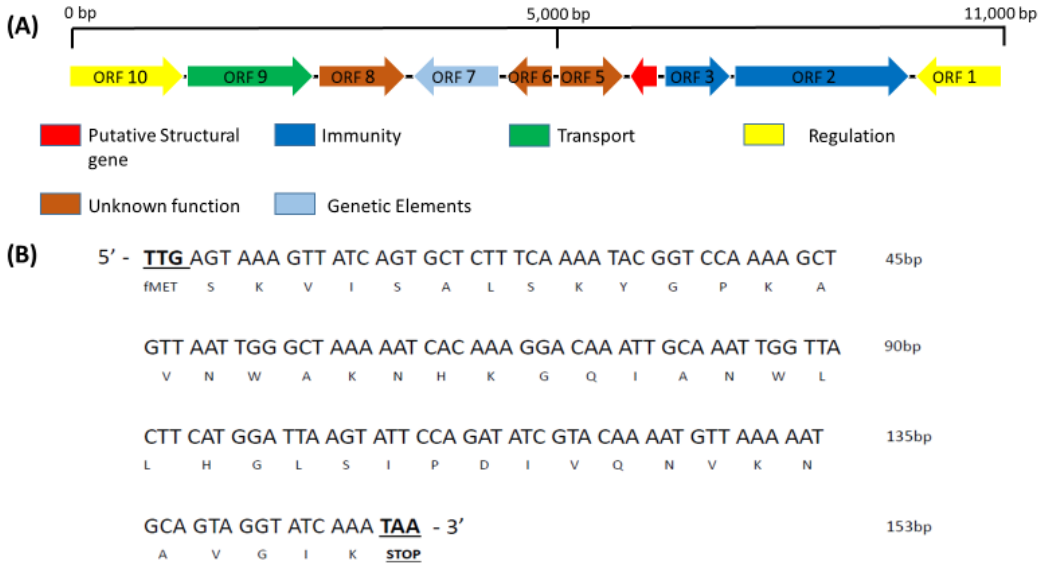

Figure 10. (A) Organisation of the genomic region that is predicted to encode capidermicin. Open reading frames (ORFs)/genes are coloured according to the predicted function. **(B)** The nucleotide sequence of the 153 bp ORF4 that is predicted to encode capidermicin. The deduced amino acid sequence is shown under the DNA sequence. The start and stop codon, TTG and TAA, respectively, are shown in bold and underlined.

Figure 11. Alignment of the capidermicin amino acid sequence with homologous bacteriocins; epidermicin NI01 from *S. epidermidis* strain 224 (JQ025383), mutacin BHT-B from *Streptococcus ratti* BHT (DQ145753), lactolisterin BU from *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 (SDR48784), lacticin Q from *L. lactis* QU5 (BAF57910), lacticin Z from *L. lactis* QU14 (BAF75975), aureocin-like produced by numerous bacteria (SEM89646), and aureocin A53 from *S. aureus* A53 (WP_010976360). Highlighted residues indicate conserved amino acid sequences.

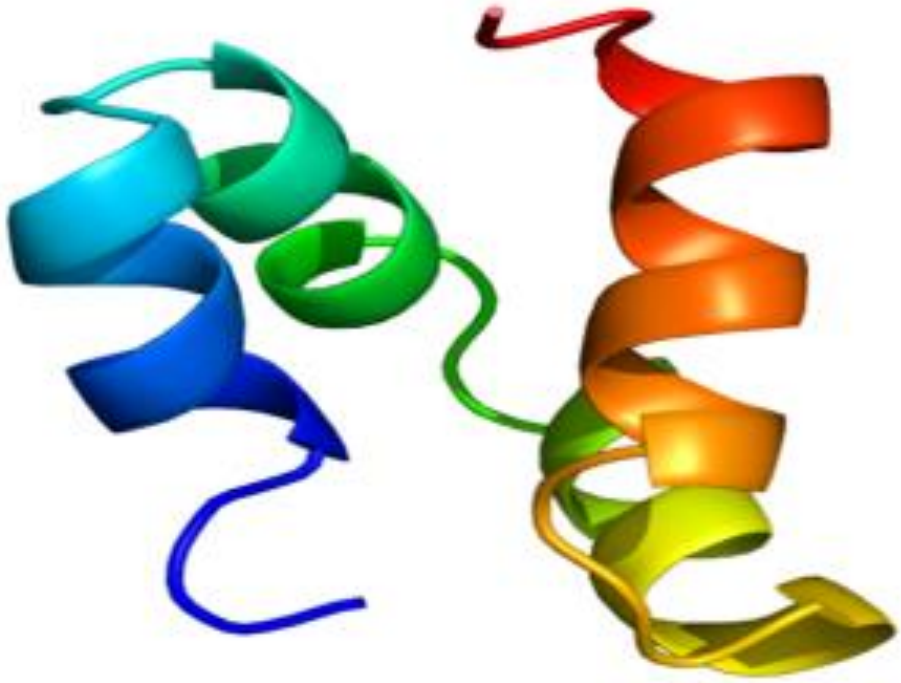

Figure 12. Putative three-dimensional structure of capidermicin. A rainbow colour scheme is used to indicate the N-terminus in blue, and the C-terminus in red. The structure was generated using Phyre2.

Chapter 3

Inhibition of *Listeria monocytogenes* by the *Staphylococcus capitis* - Derived bacteriocin capidermicin

A manuscript based upon this chapter has been published in Food Microbiology.

Lynch D, Hill C, Field D, Begley M. (2021) Inhibition of *Listeria monocytogenes* by the *Staphylococcus capitis* - derived bacteriocin capidermicin. 2020. Food Microbiology. 94, 103661. https://doi.org/10.1016/j.fm.2020.103661

3.1 Abstract

Natural methods to control food pathogens are required and bacteriocins have received much interest in this regard. The aim of this study was to investigate the ability of the novel bacteriocin capidermicin to inhibit *Listeria monocytogenes*, and important foodborne pathogen. Agar-based deferred antagonism assays were carried out with the capidermicin producer against 17 *L. monocytogenes* strains and large zones of inhibition were observed for 12 strains. Minimal inhibitory concentration assays performed with purified capidermicin peptide revealed MIC values between 680nM and 11µM. Biofilm assays were performed with five *L. monocytogenes* strains (*L. monocytogenes* CD749, EGDe, Ts45, OB001102 and F2365). Addition of capidermicin prevented biofilm formation by *L. monocytogenes* EGDe and could remove pre-established biofilms of all five strains (*L. monocytogenes* CD749, EGDe, Ts45, OB001102 and F2365). Broth based growth experiments demonstrated that addition of capidermicin resulted in an extended lag phase of both *L. monocytogenes* strains tested. Kill-curve experiments showed that capidermicin was able to potentiate the anti-*Listeria* effects of the lantibiotic nisin. The synergistic interaction of both peptides was also observed in model food systems (cottage cheese and chocolate milk). In summary, we show that capidermicin can inhibit *L. monocytogenes* and warrants further investigation as a potential natural agent for the control of this pathogen.

3.2 Introduction

A growing trend toward minimally processed and ready-to-eat (RTE) chilled food products means that more robust strategies are needed to control the growth and survival of foodborne pathogenic bacteria such as *Listeria monocytogenes* (Hanson et al., 2019; Zhao et al., 2014). Despite significant efforts to combat *L. monocytogenes* and to minimise food contamination, this pathogen still presents a significant challenge for the food industry due to its ubiquitous nature, its ability to grow at refrigeration temperatures, and the fact that it can persist during many manufacturing processes. *L. monocytogenes* is the aetiological agent of listeriosis, one of the most deadly foodborne diseases in industrialized countries (Smith et al., 2016). In the United States, *L. monocytogenes* infections are the third leading cause of death from foodborne illness and result in an estimated 1,500 infections, 1,400 hospitalizations, and 250 deaths each year (Scallan et al., 2011; Self et al., 2019). During a period from 2015 to June 2018, a Hungarian food company was the source of an outbreak that affected Austria, Denmark, Finland, Sweden and the United Kingdom. This outbreak recorded 47 listeriosis cases and nine fatalities (European Food Safety Authority and European Centre for Disease Prevention and Control, 2018). The large-scale outbreak in South Africa from 2017 to 2018, was the largest *Listeria* outbreak to date, with 1064 identified cases and greater than 200 deaths (Allam et al., 2018; Desai et al., 2019). Despite the introduction in 2006 of Food Safety Criteria (FSC) incorporating a variety of precautionary measures to control *L. monocytogenes* in RTE food products, outbreaks due to foodborne illness continue to occur periodically (European Food Safety Authority, 2015; Ricci et al., 2018). Notably, *L. monocytogenes* can form biofilms, giving it the ability to grow on multiple surfaces throughout the food processing facility, including steel, glass, rubber and polypropylene

(Galié et al., 2018; Silva et al., 2008). These biofilms are able to endure routine cleaning procedures, are resistant to disinfectants and heat (Camargo et al., 2016; Carpentier and Cerf, 2011; Pérez Ibarreche et al., 2014) and allow the organism to be a persistent contaminant.

The ability of natural antimicrobials such as bacteriocins to inhibit food-associated pathogens and spoilage bacteria has led to them receiving attention as possible alternatives to chemicals for the control of microbes in foods and food manufacturing environments (Gálvez et al., 2007). Bacteriocins are ribosomally produced cationic peptides that inhibit or prevent the growth of closely related bacterial species (Cotter et al., 2013). The best known is the lantibiotic nisin, which was approved by the US Food and Drug Administration (FDA) in 1988 because of its long safe history in food preservation and is now used in over 70 countries (Fu et al., 2018; Gharsallaoui et al., 2016). Nisin is effective against several food spoilage and pathogenic bacteria including staphylococci, bacilli, clostridia and listeria. It is used in a wide variety of foods including dairy, meat, soups, salad dressings, seafood, tomato products and beer and has recently been approved for use in unripened cheeses and heat-treated meat products (Delves-Broughton, 2005; Gharsallaoui et al., 2016). Moreover, several studies have examined nisin in combination with other antimicrobials with a view to targeting persistent food-borne microbes including *L. monocytogenes.* Nisin in combination with other naturally-derived compounds including essential oils (Ettayebi et al., 2000; Field et al., 2015b; Ndoti-Nembe et al., 2013), organic acids (Smith et al., 2016), EDTA (Wan Norhana et al., 2012) as well as other bacteriocins (Bouttefroy and Millière, 2000; Hanlin et al., 1993; Mulet-Powell et al., 1998) have been shown to target *L. monocytogenes* more effectively. For example, it was revealed that the class I/class IIa combination of nisin/curvaticin 13

was significantly more effective than either bacteriocin alone (Bouttefroy and Millière, 2000). Similarly, nisin in combination with pediocin PA-01 were observed to act in synergy against *L. monocytogenes* and a number of nisin/pediocin resistant variants (Naghmouchi et al., 2011).

We recently reported the identification and purification of a bacteriocin from the human skin-derived strain *Staphylococcus capitis* CIT060 (Lynch et al., 2019). *In silico* analyses suggests that capidermicin is a novel member of the family of class II leaderless bacteriocins; these are small peptides that contain an N-formylated methionine, are not post translationally modified and become active peptides soon after translation (Perez et al., 2018). We demonstrated that capidermicin inhibited all of the Gram-positive bacteria that were examined including members of the genera *Bacillus*, *Staphylococcus*, *Streptococcus*, *Micrococcus* and *Lactococcus*. The aim of the present study was to investigate if capidermicin could inhibit *L. monocytogenes* under standard lab conditions (broth and agar), in biofilm assays and in model food systems (cottage cheese and chocolate milk).

3.3 Materials and Methods

3.3.1 Bacterial strains and growth conditions

Staphylococcus capitis CIT060, the producer of capidermicin, and all *Listeria* isolates used in this study were obtained from the Munster Technological University (MTU) culture collection and University College Cork (UCC) culture collection, respectively (Table 5). All strains were grown in Brain Heart Infusion (BHI) broth or agar (Oxoid) at 37ºC for 18 hours, unless otherwise stated. All bacterial isolates were maintained at -20ºC in BHI containing 40% (v/v) glycerol. *Lactococcus lactis* HP and *Lactococcus lactis* MG1614 were grown in M17 broth supplemented with 0.5% glucose (GM17) (Oxoid) at 30ºC for 18 hours. For biofilm assays, strains were grown in tryptic soy broth (TSB) (Merck) supplemented with 1% glucose (Sigma-Aldrich) at 37ºC for 18 hours.

3.3.2 Agar-based deferred antagonism assays

Deferred antagonism assays for antimicrobial activity determination were performed as previously described (Field et al., 2012). Briefly, 15µl volumes of a fresh overnight culture of *S. capitis* CIT060 were spotted onto BHI agar and allowed to grow overnight. Following overnight growth, the strain was subjected to UV radiation (High performance UV transmitter, Upland, Ca, USA) for 30 minutes. The listerial indicator was seeded at 0.1% (v/v) into 20 ml soft BHI agar (BHI broth supplemented with 0.75% agar), poured over the test plate and incubated at 37ºC for 16 – 18 hours.

3.3.3 Recovery and purification of capidermicin from S. capitis CIT060

Capidermicin was purified from *S. capitis* CIT060 as previously described in Chapter 2 (Lynch et al., 2019). Briefly, three litres of clarified BHI (cBHI) broth were incubated for 18-20 hours at 37^{o}C with a 1% inoculum of *S. capitis* CIT060. Following incubation, the culture was centrifuged for 20 minutes at 7,000 x *g*. The cell-free supernatant (CFS) was removed, collected, and passed through 60g of pre-equilibrated Amberlite XAD16N beads (Sigma Aldrich) with subsequent washing of the column with 500 ml of 30% ethanol and elution in 400 ml of 70% isopropanol (IPA) (Fisher) with 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich) The CFS eluate was reduced by rotary-evaporation (Buchi, Switzerland) to approximately 250 ml. The sample was further purified and concentrated to 60 ml through a Phenomenex SPE C-18 column (Phenomenex, Cheshire, UK). Purification of the peptide was achieved using a Phenomenex C12 Reverse-Phase (RP) HPLC column (Jupiter 4 µ proteo 90 Å, 250 X 10.0 mm, 4 µm). The RP-HPLC column was equilibrated with 25% acetonitrile (ACN) – 0.1% TFA. Subsequently, a gradient of 25-50% ACN containing 0.1% TFA was developed at a flow rate of 2 ml/min. Collection and pooling of active fractions was followed by removal of ACN by rotary evaporation and freeze-drying. Well diffusion assays with the indicator strain *L. lactis* HP were employed at all stages of the purification protocol to monitor bacteriocin activity. The purity of the peptide was analysed through MALDI-TOF mass spectrometry.

3.3.4 Purification of nisin from *L. lactis* NZ9700

Nisin A was purified following the method described previously (Field et al., 2019). Briefly, the nisin A producing strain *L. lactis* subsp. *lactis* NZ9700 was subcultured twice at 30°C for 20 hours in GM17 broth prior to use. A 0.5% (v/v) inoculum was added to modified tryptone yeast (TY) broth (2 Litres) and incubated at 30°C overnight. The

culture was centrifuged at 7,000 x *g* for 20 min. The cell-free supernatant was removed, collected, and passed through 60g of pre-treated Amberlite XAD16N beads (Sigma Aldrich). Following a 30% ethanol wash step, the active fraction was eluted in 400 ml 70% IPA + 0.1% TFA. The cell supernatant was further concentrated through rotary-evaporation to approximately 250 ml. Cells were resuspended in 250 ml of 70% IPA + 0.1% TFA and agitated by magnetic stirrer for 3 hours at room temperature. Cellular debris was separated by centrifugation at 6500 g for 15 min and the supernatant retained. The supernatant and cell pellet eluates (approximately 650 ml) were pooled and the 70% IPA + 0.1% TFA was removed by rotary evaporation (Buchi, Switzerland). The sample pH was adjusted to 4 prior to application to a 10 g (60 ml) Varian C-18 Bond Elution Column (Varian, Harbor City, CA) pre-treated with methanol and water. Following wash steps with 60 ml of 30% ethanol, the active fraction was extracted in 60 ml of 70% IPA + 0.1% TFA. 12 ml aliquots were subject to rotary evaporation to remove the isopropanol to a final volume of 2 ml. 2 ml aliquots were applied to a Phenomenex (Phenomenex, Cheshire, UK) C12 reverse phase (RP)-HPLC column (Jupiter 4u proteo 90 Å, 250 × 10.0 mm, 4 µm) previously equilibrated with 25% ACN + 0.1% TFA. The column was subsequently developed in a gradient of 25 % ACN + 0.1% TFA to 50% ACN containing 0.1% TFA from 10 to 40 min at a flow rate of 3.2 ml min^{-1}. Relevant fractions were collected and pooled, the acetonitrile was removed by rotary evaporation and water was removed by freeze-drying. The purity of the peptide was analysed through MALDI-TOF mass spectrometry (Cotter et al., 2006).

3.3.5 Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) assays were carried out in 96 well microtitre plates in triplicate as described by Field et al., (2015b). Briefly, test wells within the 96 microtitre plate (Sarstedt, Leicester, UK) were pre-treated with 200 µl phosphate buffered saline (PBS) supplemented with 1% (w/v) bovine serum albumin (PBS/BSA). The plates were incubated for 30 min at 37°C. Removal of the PBS/BSA was followed by washing with PBS (200 µl) and plates were subsequently allowed to air-dry. Freshly grown strains of *L. monocytogenes* were transferred into fresh BHI broth and incubated to reach an OD_{600} of 0.5 [$\sim 10^7$ colony forming units (CFU)/ml] and then diluted to reach a final concentration of $\sim 10^5$ CFU/ml. Purified capidermicin peptide was reconstituted in BHI broth, and 2 - fold serial dilutions carried out using a multi-channel pipette to reach 12 dilutions in total. The strain of interest was then added and the plates incubated at 30^{o}C or 37^{o}C for 16 hours. The MIC was read as the lowest peptide concentration causing inhibition of visible growth. MIC determinations were conducted using biological triplicates.

3.3.6 Biofilm formation

L. monocytogenes biofilm assays were performed as previously described (Kelly et al., 2012), but with modifications. TSB (Merck) broth was supplemented with 1% D-(+)-glucose (Sigma) (TSBg) to improve *L. monocytogenes* biofilm formation (Choi et al., 2013). 2 µl of each overnight culture were added to 198 µl of TSBg in separate wells of a sterile 96-well microtitre plate (Sarstedt, Leicester, UK). Microtitre plates were then incubated at 37°C for 24 h to allow biofilm formation to occur. Following incubation for 24 hours at 37^{o}C, the plates were removed and gently washed with PBS, then 200 µl methanol was placed in the wells for 15 minutes. The methanol was removed and 200 µl

of 0.05% crystal violet was added to each well for 15 minutes. The crystal violet was then removed, and 100µl of 33% acetic acid was placed in each well. Absorbances were measured at 595nm using a microtitre plate reader, SpectraMax M3 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A well containing TSBg alone served as a negative control. Assays were carried out in triplicate with three biological repeats. Biofilm results are presented as the mean ± SD.

3.3.7 Biofilm prevention with purified capidermicin

The ability of capidermicin to prevent *L. monocytogenes* biofilms was carried out as described above with the following modifications. 2 µl of each overnight culture was added to 198 µl of TSBg in separate wells of a sterile 96-well microtitre plate (Sarstedt, Leicester, UK). Capidermicin peptide was added at concentrations 2X, 1X, 1/2X, 1/4X, 1/8X and 1/16X MIC to the microtitre plate wells and incubated at 37^{o}C for 24 hours. Wells containing *L. monocytogenes* cells without added peptide served as untreated controls. Wells containing TSBg alone served as a negative control. After 24 hours, the plate was removed and gently washed once with PBS, fixed with methanol, stained with 0.05% crystal violet, 100µl of 33% acetic acid was placed in each well as described previously, and optical density readings were taken at 595 nm (OD595) to determine the final biofilm biomass. All tests were conducted using three biological repeats.

3.3.8 Biofilm treatment with purified capidermicin

Biofilm formation was carried out as described earlier. Following incubation for 24 hours, pre-established biofilms were washed once with phosphate buffered saline (PBS). To each test well of the microassay plate capidermicin peptide was at 1X, 2X, 4X, 8X and 16X of the relevant MIC. All wells were seeded in triplicate. The microtitre plates were incubated for 24 h at 37°C, and subsequently washed once with PBS, dehydrated with

methanol, stained with 0.05% crystal violet and 100µl of 33% acetic acid was placed in each well as described previously. Finally, optical density readings were taken at 595 nm (OD595) to determine the final biofilm biomass. All tests were carried out using three biological repeats.

3.3.9 Growth and kill curve experiments

For growth assays, overnight cultures of *L. monocytogenes* EGDe and F2365 were diluted to a final concentration of 10^5 CFU/ml in a volume of 1.0 ml BHI broth containing capidermicin at concentrations 4X, 2X, 1X, 1/2X, 1/4X and 1/8X MIC of the test strain. 200 µl were transferred to a 96-well microtitre plate and absorbance readings taken (at OD_{600}) using a Spectra Max 340 spectrophotometer (Molecular Devices, Sunnyvale, California) over a period of 24 hours at 37ºC. For time-point kill experiments, fresh overnight cultures of *L. monocytogenes* EGDe and F2365, were diluted (final concentration of 10^5 CFU/ml in a volume of 1.0 ml) in BHI broth containing the relevant concentration of capidermicin alone (1X MIC) or in combination with nisin A (1X and 1/2X MIC) against the test strain. Samples were incubated for 3 hours at 37°C. Bacterial cell numbers were measured by performing viable cell counts by diluting cultures in one-quarter-strength Ringers' solution and enumerating on BHI agar plates. All tests were conducted using three biological repeats.

3.3.10 Model food studies

3.3.10.1 Cottage cheese trial

An aliquot of a commercially available cottage cheese was streaked onto *Listeria* Selective Agar (LSA) (Lab M) to ensure the absence of intrinsic contaminating *L. monocytogenes*. The pH of the cottage cheese was determined to be 4.6. Triplicate samples of the cottage cheese (1 gram) were homogenized using a stomacher (IUL

Instruments, Barcelona, Spain) in 9 ml ¼ strength Ringers' solution (Merck). Upon the addition of Ringers' solution, the pH of the cottage cheese was re-tested and a pH of 4.66 was recorded. Overnight cultures of *L. monocytogenes* EGDe or F2365 were diluted and inoculated into the cottage cheese at a final concentration of 10^5 CFU/ml. To the cottage cheese containing EGDe, capidermicin was added at a concentration of 3.75 µg/ml (1X MIC), and nisin was added at a concentration of 12.57 µg/ml (1X MIC) or 6.25 µg/ml (1/2X MIC). For *L. monocytogenes* F2365, capidermicin and nisin A were each added at a concentration of 15 µg/ml (1X MIC) or 7.5 µg/ml (1/2X MIC). The cottage cheese was incubated at 22ºC (room temperature) for 3 hours, samples were collected after 1 and 3 hours and counts of *Listeria* were determined through serial dilutions and spread plating onto LSA. All experiments were carried out in triplicate.

3.3.10.2 Chocolate milk trial

A supermarket bought chocolate milk product was streaked onto *Listeria* Selective Agar (LSA) (Lab M) to ensure the absence of contaminating *L. monocytogenes*. The pH of the chocolate milk was determined to be 6.85. Aliquots of chocolate milk were aseptically transferred to 1.5 ml Eppendorf tubes. *L. monocytogenes* EGDe and F2365 were inoculated into the chocolate milk at a final concentration of 10^5 CFU/ml. Capidermicin alone and in combination with nisin A were each added to the Eppendorf tubes at 1X and 1/2X the relevant MIC. The chocolate milk was incubated at 22ºC (room temperature) for 3 hours, samples were collected after 1 and 3 hours and counts of *Listeria* were determined through serial dilutions and spread plating onto LSA. All experiments were carried out using biological triplicates.

3.3.11 Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) using IBM SPSS V.25 statistical software. In all cases, a *P* value = 0.05 was considered statistically significant. Asterisks *, ** or *** indicate statistically significant differences between samples (*P* = 0.05, *P* = 0.005 and *P* = 0.001, respectively). All CFU/ml data were transformed to $\log_{10}$ prior to the use of statistical software package. All comparisons between data were based on the mean ± standard deviation.

3.4 Results

3.4.1 Overlay assays and minimum inhibitory concentrations (MICs)

Seventeen *L. monocytogenes* strains were tested for susceptibility to capidermicin using agar-based deferred antagonism assays. Zones of inhibition were observed for twelve strains (Fig. 13). To more accurately assess the activity of capidermicin against the bank of *L. monocytogenes* strains, broth-based MIC determination assays were carried out using purified capidermicin (Table 5). The MIC value was determined to be the lowest concentration of capidermicin that resulted in the absence of visible growth of the target *Listeria* strain after 16 h at 37°C in BHI broth. *L. monocytogenes* strains EGDe, Ts45, CD749, OB001102 and LO28 proved to be the most sensitive to capidermicin (Table 5), with *L. monocytogenes* EGDe exhibiting the lowest MIC value of 3.75 µg/ml (680 nM). Three *L. monocytogenes* isolates G6003, F1109 and CD1028 displayed the highest MIC values (>60 µg/ml or >11µM). In contrast, food epidemic strains F2365 and H7858, and a clinical isolate F5817, were more sensitive to capidermicin with an observed MIC value of 15 µg/ml (2.75 µM).

3.4.2 Investigation of the anti-biofilm activity of capidermicin

In view of the inhibitory effect of capidermicin against planktonic cells of *Listeria*, we sought to determine if it could be effective in the prevention of biofilm formation or in the eradication of established biofilms. While *L. monocytogenes* is a known biofilm former, significant strain-to-strain variation in its ability to form biofilms has been reported (Colagiorgi et al., 2017). Consequently, prior to initiating these studies, we evaluated the biofilm forming abilities of *L. monocytogenes* CD749, EGDe, Ts45, OB001102 and F2365. All five strains demonstrated an ability to form biofilms when assessed by crystal violet staining in microtitre plate assays. For biofilm prevention

studies, a starting concentration of 2X MIC of capidermicin and subsequent 2-fold dilutions to 1/16th MIC was chosen. Subsequently, the listerial strain of interest was added prior to incubation at 37°C for 24 hrs. Following staining and optical density readings at 595 nm (OD_{595}), a significant reduction in *L. monocytogenes* EGDe biofilm biomass (*P* value = 0.05) was observed in wells containing 2X (7.5 µg/ml), 1X (3.75 µg/ml) and 1/2X (1.875 µg/ml) MIC compared to the untreated control (Fig. 14). When lower concentrations 1/4X (0.9375 µg/ml), 1/8X (0.468 µg/ml) and 1/16X (0.234 µg/ml) MIC was used, significant reductions in biofilm density were not observed when compared to the untreated control. For the remaining *L. monocytogenes* isolates tested; CD749, Ts45, OB001186 and F2365, capidermicin was not able to prevent biofilm formation at any of the concentrations utilised (Fig. 14). It was notable that EGDe formed the poorest biofilms of all five strains tested, which may explain why capidermicin was able to inhibit this strain more effectively.

The efficacy of capidermicin to eradicate established biofilms of *L. monocytogenes* CD749, EGDe, Ts45, OB001102 and F2365 was investigated. Pre-formed biofilms in 96-well plates were exposed to capidermicin at concentrations of 1X, 2X, 4X and 8X MIC for 24 hours, followed by crystal violet staining and optical density readings at 595 nm (Fig. 15). For *L. monocytogenes* CD749, a statistically significant reduction (*P* value = 0.001) in biofilm mass was obtained at 2X, 4X and 8X MIC (15 µg/ml, 30 µg/ml and 60 µg/ml, respectively) (Fig. 15A). Similarly, a statistically significant reduction in biofilm biomass was observed for *L. monocytogenes* Ts45 (epidemic outbreak isolate) (*P* value = 0.001) at 4X and 8X MIC only (30 µg/ml and 60 µg/ml, respectively) (Fig. 15C). In the case of *L. monocytogenes* F2365, significant loss of biofilm was observed at all concentrations tested (1X (*P* value = 0.013), 2X (*P* value

= 0.007), 4X (*P* value = 0.001) and 8X MIC (*P* value = 0.001) when compared with the untreated control (Fig. 15E)). When treated biofilms of *L. monocytogenes* EGDe were examined (Fig. 15A), reductions in biofilm mass were observed at capidermicin concentrations of 2X and 4X MIC with the greatest reduction observed at 8X MIC (30 µg/ml). For *L. monocytogenes* OB001102, a decrease in biofilm mass was observed at 1X, 2X, 4X and 8X MIC (7.5 µg/ml, 15 µg/ml, 30 µg/ml and 60 µg/ml, respectively), with 8X generating a statistically significant reduction (*P* value = 0.001) when compared to the untreated control.

3.4.3 Growth curve assays

To expose the finer details of the effectiveness of capidermicin on cell viability that are not readily observed from end-point MIC determinations and biofilm assays, growth curve analysis was undertaken to investigate the impact of increasing concentrations of capidermicin on *L. monocytogenes*. *L. monocytogenes* EGDe and F2365 were selected as representative test strains for growth curve experiments (Fig. 16A and 16B, respectively). For *L. monocytogenes* EGDe, capidermicin concentrations 1/8X MIC, 1/4X MIC 1/2X and 1X displayed growth rates similar to that of the untreated control (data not shown). For 2X and 4X MIC peptide concentrations, bacterial growth was significantly retarded, and a progressively extended lag time was observed (Fig. 16A). For *L. monocytogenes* F2365 growth curves a similar result was observed, with 1/8X MIC, 1/4X MIC 1/2X and 1X, having growth rates similar to that of the untreated control (data not shown). The capidermicin concentrations 2X and 4X MIC also caused an extended delay in growth when compared to the untreated control (Fig. 16B). Growth curve analysis revealed that for both *L. monocytogenes* strains, growth rates decreased in the presence of increasing

concentrations of capidermicin. These findings may suggest that when acting alone capidermicin is bacteriostatic.

3.4.4 Assessment of capidermicin and nisin A alone and in combination against *Listeria*

Next, we sought to determine the effectiveness of capidermicin in laboratory media in combination with nisin A. The MIC of nisin for *L. monocytogenes* EDGe and F2365 was determined to be 12.57 and 15µg/ml, respectively, which is in agreement with our previous studies (Campion et al., 2013; Field et al., 2010b). The addition of 1X MIC capidermicin resulted in a 0.8 log increase in *L. monocytogenes* EGDe cell numbers after the three hour incubation period (Fig. 16C). When capidermicin and nisin A were used in combination against *L. monocytogenes* EGDe at 1X and 1/2X MIC (3.75 µg/ml and 6.29 µg/ml), respectively, a slight increase in bacterial cell counts was observed when compared to the starting inoculum (Fig. 16C). In contrast however, treatment with 1X MIC capidermicin + 1X MIC nisin A (3.75 µg/ml and 12.57 µg/ml, respectively) resulted in an approximate 2.3-log reduction in *L. monocytogenes* EGDe cell counts. A similar pattern was observed for *L. monocytogenes* F2365, whereby the capidermicin treatment alone as well as the combination with nisin A at 1X capidermicin MIC and 1/2X nisin MIC concentrations (15 µg/ml and 7.5 µg/ml, respectively), did not significantly reduce cell numbers (Fig. 16D). Notably, a statistically significant 1.25-log reduction (*P* value = 0.001) in bacterial cell counts was observed for the peptide combination (1X MIC + 1X MIC) (Fig. 16D).

3.4.5 Model food studies

Previous studies have revealed that bacteriocin efficacy can be severely impacted within a food matrix as a result of several factors that include adsorption to fat or sodium chloride

concentrations (Gálvez et al., 2007). Therefore, having established the efficacy of the capidermicin and nisin A combinations against *L. monocytogenes* in standard laboratory assays, it was decided to examine if this enhanced effectiveness would also be observable in a food environment. Two food trials were conducted using a commercially manufactured cottage cheese and a commercially produced chilled chocolate milk drink. These food products were chosen in light of their previous association with *L. monocytogenes* outbreaks (Hanson et al., 2019; Pearson and Marth, 1990). For all experiments, cottage cheese or chocolate milk was aseptically transferred to containers to which purified capidermicin (1X MIC) or nisin A (1X MIC) or a combination of both (1X + 1X and 1X + 1/2X MIC, respectively) was directly added.

In the cottage cheese model food trial with *L. monocytogenes* EGDe (Fig. 17A), plate counts revealed that cell numbers remained unchanged in the presence of capidermicin alone after one hour and had increased by 1 log after 3 hours. In the presence of nisin A alone, a ~1.7-log reduction (*P* value = 0.015) in CFU/ml was observed after one hour and 3 hours of treatment (Fig. 17A). Similarly, the capidermicin and nisin combination at 1X + 1/2X MIC (3.75 µg/ml and 6.29 µg/ml, respectively) led to a reduction of 1.8-logs after 1 hour, though bacterial numbers had regained slightly after 3 hours (Fig. 17A). The peptide combination at 1X + 1X MIC (3.75 µg/ml and 12.57 µg/ml, respectively) produced a ~2.6 log reduction in bacterial cell numbers (*P* value = 0.001), no further detectable change in cell counts was observed following three hours of incubation (Fig. 17A). In chocolate milk with capidermicin alone added, plate counts revealed no change in *L. monocytogenes* EGDe numbers for one hour and an increase in CFU/ml after three hours (Fig. 17B). With respect to nisin A alone, a ~1.2 log (*P* value = 0.001) reduction was achieved. Notably, the peptide combination (1X capidermicin + 1X

nisin A MIC; 3.75 µg/ml and 12.57 µg/ml, respectively) proved to be the most effective treatment and brought about a statistically significant 2.5 log reduction (*P* value = 0.001) against *L. monocytogenes* EGDe after one hour, which remained at ~2.5 log CFU/ml (*P* value = 0.001) after three hours incubation (Fig. 17B).

A similar pattern to that of *L. monocytogenes* EGDe was discerned in the cottage cheese experiment involving *L. monocytogenes* F2365 (Fig. 17C). Though nisin A alone produced a statistically significant two log reduction (*P* value = 0.012) in CFU counts after 1-hour, bacterial numbers had increased by two logs following a further two hours of incubation (Fig. 17C). The peptide combination of 1X MIC capidermicin + 1X MIC nisin A delivered a significant 2.5 log (*P* value = 0.012) CFU/ml reduction after one hour. Moreover, after three hours, the listerial numbers were observed to diminish further (~3.0 log reduction), when compared to the initial starting inoculum (Fig. 17C). In contrast, cell numbers were observed to increase further in the presence of the peptide combination at 1X capidermicin and 1/2X nisin.

When the peptides were assessed in chocolate milk, in the presence of capidermicin at 1X MIC, cell counts of *L. monocytogenes* F2365 (Fig. 17D) remained unchanged. Little difference was observed between nisin alone (0.5 log CFU/ml reduction) or nisin + capidermicin combinations (1X MIC capidermicin + 1X MIC nisin A (~0.6 log CFU/ml reduction) and 1X MIC capidermicin + ½ MIC nisin A (~0.4 log CFU/ml reduction) after one hour of treatment. Moreover, bacterial cell counts were observed to increase significantly after three hours in the peptide and peptide combination treatments (Fig. 17D).

3.5 Discussion

Despite best efforts, recent and frequent food-associated outbreaks of *L. monocytogenes* have demonstrated that current food preservation methods can be sub-optimal in preventing listerial contamination in food (Allam et al., 2018; Hanson et al., 2019). Consequently, the identification of novel, natural and effective strategies to control *L. monocytogenes* would be of considerable importance and benefit to food manufacturers and consumers. Antibacterial peptides known as bacteriocins could form the basis of one such strategy. Capidermicin is a recently characterised class II leaderless bacteriocin produced by *S. capitis* CIT060 (Lynch et al., 2019). Specific activity studies revealed that capidermicin was active in the micro-molar range against a range of Gram-positive bacteria, including *Lactococcus*, *Streptococcus* and *Staphylococcus*. In this study we demonstrate that capidermicin can inhibit *L. monocytogenes*; MIC ranging from 3.75 - >60 ug/ml were obtained. Other class II peptides homologous to capidermicin e.g. lacticin Q and aureocin A53, have been shown previously to inhibit *Listeria* strains (Fagundes et al., 2016; Fujita et al., 2007). Indeed, lacticin Q exhibited MIC values in the low nanomolar range against *L. innocua* ATCC 33090 and aureocin A53 demonstrated activity against nine *L. monocytogenes* strains.

Variances in resistance of *L. monocytogenes* strains to antimicrobial peptides including bacteriocins has previously been reported and may be as a result of differences in membrane lipid composition, cell membrane thickness or charge (Begley et al., 2006).Genetic loci that have been linked to bacteriocin resistance include the *dlt*ABCD operon that catalyses the incorporation of D-alanine into cell wall-associated lipoteichoic acids (Abachin et al., 2002), *mprF* that is involved in lysinylation of membrane phospholipids (Henderson et al., 2020), the tellurite resistance gene *telA* (Collins et al.,

2010b) and the *anrAB* locus that encodes an ABC transporter (Collins et al., 2010a). To our knowledge the role of these aforementioned loci in strain variable bacteriocin resistance has not been examined. The glutamate decarboxylase (Gad) system plays a role in the tolerance of low pH and has been the focus of several studies in our laboratory. *L. monocytogenes* can possess five gad genes at three separate genetic loci: *gad*D1T1, *gad*D2T2 and *gad*D3 (Paul D. Cotter et al., 2005). We initially reported that while all of the *L. monocytogenes* strains that we examined possessed *gad*D2, *gad*T2 and *gad*D3 genes, *gad*D1 that encodes a glutamate decarboxylase and its' associated transporter gadT1 were strain variable i.e. *gad*D1T1 was not present in all strains (Paul D. Cotter et al., 2005; Ryan et al., 2010). We subsequently demonstrated that a *gad*D1 mutant was significant more sensitive to nisin that its counterpart wildtype strain (Begley et al., 2010). In addition, we observed that the presence of gadD1 in a collection of *L. monocytogenes* strains correlated with a higher degree of tolerance to nisin i.e. the natural presence or absence of *gad*D1 impacted the ability of individual strains to tolerate nisin (Begley et al., 2010). The relationship between the presence of absence of *gadD1* and the observed variation in innate resistance of *L. monocytogenes* strains to capidermicin has not been investigated.

L. monocytogenes has been shown to form biofilms in food processing environments. As a consequence of the extensive resistance of listerial biofilms to established treatments including biological and chemical sanitizers, numerous studies have explored the ability of various bacteriocins to inhibit adhesion and biofilm formation by *L. monocytogenes* on different abiotic substrates. The bacteriocins examined include nisin (Bolocan et al., 2016; Bower et al., 1995), enterocins (Caballero Gómez et al., 2012), sakacin 1 (Winkelströter et al., 2011) and one class II leaderless bacteriocin (Cirkovic et

al., 2016). The results reveal that bacteriocins may delay but do not completely prevent biofilm formation by *L. monocytogenes* (Rodríguez-López et al., 2018). Similarly, capidermicin was unable to completely prevent biofilm formation for any of the listerial isolates at even the highest concentrations used (2X MIC). Notably, significant reductions in established biofilm mass were observed against all the *L. monocytogenes* strains tested (CD749, EGDe, Ts45, OB001102 and F2365) at 4X and 8X MIC. Indeed, studies with the capidermicin homolog lacticin Q revealed that it was capable of killing biofilm cells of *S. aureus* at 4X MIC, resulting in a one log reduction in CFU/ml counts (Okuda et al., 2013) but could not completely kill all biofilm cells after a 24-h incubation. Moreover, it was demonstrated that lacticin Q was able to penetrate the biofilm matrix and reach the deepest part of the biofilm. Although assessment of the biofilm treatments in this study were carried out utilising crystal violet assays which does not give an indication of the viability of the remaining biofilm cells, it is evident that capidermicin is capable of partial clearing of established biofilms from the surface and thus has potential as a novel antibiofilm agent.

While some studies have uncovered particularly favourable synergistic relationships between different bacteriocins (Hanlin et al., 1993; Jamuna et al., 2005; Jamuna and Jeevaratnam, 2009; Vignolo et al., 2000), given the copious extent of bacteriocin-antimicrobial permutations that remain to be examined , there potentially exists valuable combinations against targeted pathogens which are currently untapped (Mathur et al., 2018, 2017). Here, we demonstrate the ability to provide even greater protection against *L. monocytogenes* by combining for the first time a class II leaderless bacteriocin with the class I lantibiotic nisin. In time-kill assays, a two-log decrease in cell numbers over and above that achieved by the nisin A control was observed against both

L. monocytogenes EGDe and F2365. The fact that this interaction was shown to be maintained in a food matrix is noteworthy and highlights the potential of bacteriocin combinations when utilized in multi-hurdle systems to effectively control *L. monocytogenes* in food. Apart from the human health risk, the costs associated with contaminated food products due to *L. monocytogenes* are a significant financial burden, with a 2008 outbreak in Canada estimated to have a total cost of $242 million Canadian dollars (Thomas et al., 2015). In the United States, the CDC estimates an average of 1,600 cases of foodborne listeriosis per year (Scallan et al., 2011). The total estimated annual cost of listeriosis in the United States, including medical expenses, productivity losses and death, is $2.6 billion dollars per year (Hoffmann et al., 2015; Thomsen and McKenzie, 2001). Therefore, any new food preservation methods to control *L. monocytogenes* contamination are very desirable. The consistency of the capidermicin and nisin combination to control the *L. monocytogenes* isolates in two different food systems is remarkable, especially since the level of capidermicin used appears to be bacteriostatic rather than bactericidal. Additionally, the fact that this bacteriocin association is maintained in a food setting is a key finding given that decreased effectiveness can occur as a result of peptide interactions with food constituents including protein, fat, salt and the degree of bacterial contamination (Favaro et al., 2015). It is also worth noting that our study employed a high initial bacterial inoculum (1×10^5 CFU/ml) which greatly exceeds that which would be expected to be present in a food product (≈10 - 20 CFU/g). It is however important to highlight the limitations of our experiments. The two strains investigated were not the most resistant to capidermicin as determined by MICs (Table 5). In addition, the timeframe of our experiments was short (i.e. hours) so was focused on looking at the immediate effects of the bacteriocin combinations. Future

investigations should include a larger collection of L. monocytogenes strains and be carried out over the commercial shelf life of the foods (i.e. days rather than hours).

The use of nisin A is likely to increase in coming years because of increased consumer desire for foods that are minimally processed and lack artificial additives, and the fact that nisin A is one of only two natural preservatives to meet the criteria for addition to the European Union (EU) food additive list. Although nisin has numerous applications in a variety of food products, resistance to nisin by *L. monocytogenes* has been reported, prompting the search for novel alternative antimicrobials, either to replace or to be used in combination with nisin as a food preservative. Indeed, there are encouraging reports of the effective control of food-borne pathogens with other natural antimicrobials (Field et al., 2015b, 2012; Mills et al., 2017; O'Shea et al., 2010). Moreover, the ability of at least one leaderless bacteriocin has been shown to be effective in the inhibition of *L. monocytogenes* in food when Fagundes and colleagues (Fagundes et al., 2016) revealed aureocin A53, produced by *S. aureus* A53, to be highly lytic against *L. monocytogenes* in skimmed milk. Importantly, many leaderless bacteriocins do not exhibit cytotoxic and haemolytic activities, a key feature increasing their safety in food applications (Fagundes et al., 2016; Sandiford and Upton, 2012).

Although the sensitivity of *L. monocytogenes* to nisin has been documented, the antimicrobial action of nisin can vary distinctly depending on the food matrix and under various environmental conditions (Field et al., 2015b, 2008; Rouse et al., 2012). The use of capidermicin in conjunction with nisin A could provide more effective control of *L. monocytogenes* and may prevent resistance development, which might occur due to repeated use of nisin A. Therefore, the combinations of peptides as used in this study exemplify the concept of hurdle technology whereby bacterial growth is unable to

surmount the combined effects of dual acting antimicrobials. Nisin A and capidermicin represent different classes of bacteriocins (class I and class IId, respectively) and are distinctive in terms of structure, molecular weight, and mode of action. Consequently, the combination of these antimicrobials may enable lower concentrations of the individual components in suitable food products. Additionally, previous studies have also shown that capidermicin is stable at high temperatures and under acidic conditions (Lynch et al., 2019) making it ideal for use in processed low pH foods. Additional studies will be required to fully realise the potential application of capidermicin for use in food. Paramount will be the elucidation of the apparent synergistic relationship with nisin as a food preservative and potentially, combinations with bioengineered nisin derivatives which have been shown to possess enhanced activity against *L. monocytogenes* compared to nisin A that could further enhance the safety of foods (Field et al., 2010b; Rouse et al., 2012). Ultimately, combinations of natural antimicrobials such as those described in this study with other minimal food manufacturing processes within the hurdle concept could prove invaluable to commercial food producers in their quest to provide consumers with safe food with an extended shelf life without affecting nutritional properties.

Acknowledgements

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Table 5. Minimum inhibitory concentration (MIC) values of purified capidermicin against a range of *L. monocytogenes* indicators. For each strain, identical MICs values were obtained in three independent determinations.

Strain name	Source	Lineage	Serotype	Capidermicin MIC
F2365	California, U.S., food outbreak, 1985	I	4b	15 µg/ml (2.75 µM)
EGDe	Guinea pig, Cambridge, England	I	4b	3.75 µg/ml (680 nM)
LO28	Clinical isolate (Faeces of healthy pregnant woman)	I	4b	7.5 µg/ml (1.37 µM)
N3-022	Halifax, Canada, food outbreak, 1981	I	4b	30 µg/ml (5.5 µM)
H7858	U.S. Food outbreak, 1998-1999	I	4b	15 µg/ml (2.75 µM)
G6003	Food	I	1/2b	>60 µg/ml (>11 µM)
F1109	Food	I	4b	60 µg/ml (>11 µM)
RM2387	Food	I	4b	30 µg/ml (5.5 µM)
OB001186	Food	I	1/2b	30 µg/ml (5.5 µM)
Ts45	United Kingdom, Food outbreak, 1988	I	4b	7.5 µg/ml (1.37 µM)
CD749	Ground Beef	I	non-4	7.5 µg/ml (1.37 µM)
CD1121	Ground Beef	I	4b	30 µg/ml (5.5 µM)
CD1061	Pork Sausage	II	non-4	15 µg/ml (2.75 µM)
OB001102	Food	I	1/2b	7.5 µg/ml (1.37 µM)
RM2218	Food	I	4b	30 µg/ml (5.5 µM)
CD1028	Pork sausage	I	non-4	>60 µg/ml (>11 µM)
F5817	Massachusetts, clinical isolate, 1983	I	non-4	15 µg/ml (2.75 µM)

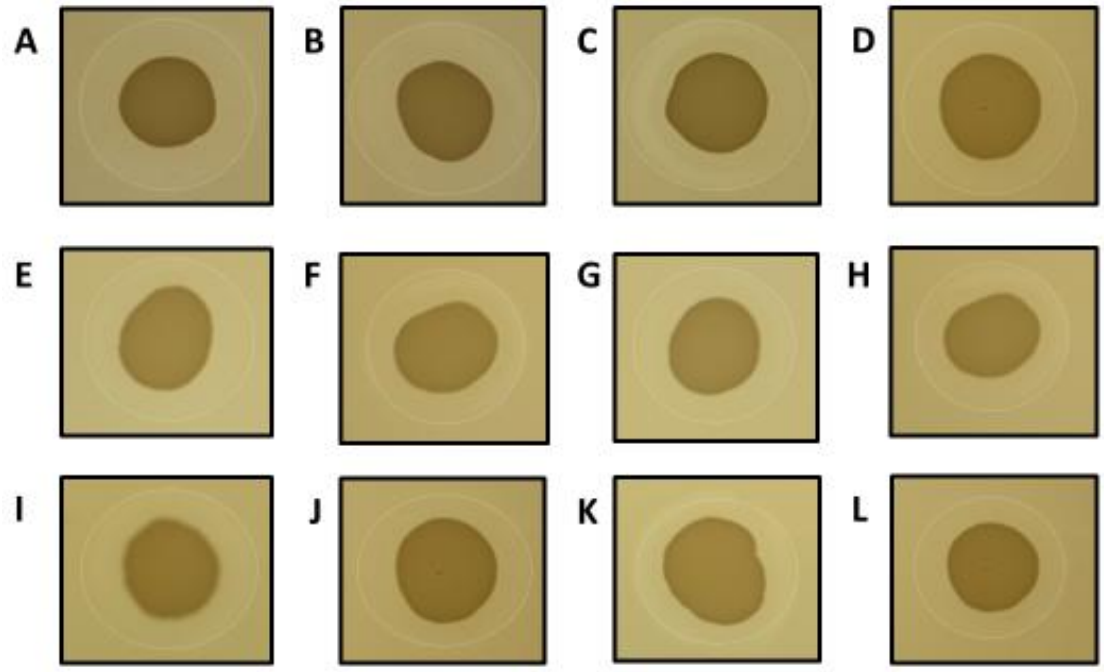

Figure 13. Deferred antagonism assay of *S. capitis* CIT060 against *L. monocytogenes* (A) EGDe, (B) F2365, (C) LO28, (D) N3-022, (E) H7858, (F) RM2387, (G) OB001186, (H) Ts45, (I) CD749, (J) CD1061, (K) OB001102 and (L) F5817. 15µl of an overnight culture of *S. capitis* CIT060 was spotted onto BHI agar plates. The plates were incubated at 37ºC overnight after which they were overlayed with sloppy BHI agar containing the relevant Listeria strain and incubated again at 37°C overnight.

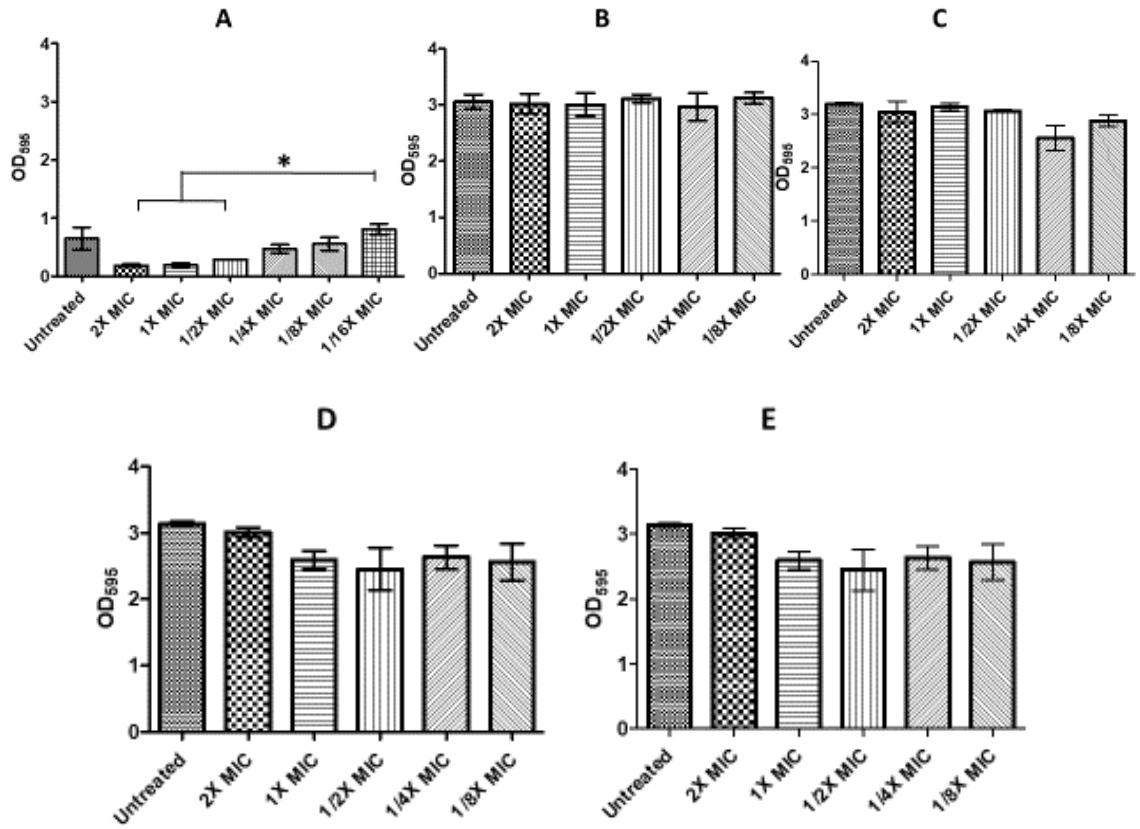

Figure 14. Inhibition of *L. monocytogenes* biofilm formation with capidermicin. Inhibition of biofilm formation of *L. monocytogenes* (A) EGDe, (B) F2365, (C) CD749, (D) Ts45 and (E) OB001102 with 2X, 1X, 1/2X, 1/4X, 1/8X and 1/16X MIC capidermicin peptide for 24 hrs as evaluated by crystal violet staining. The amount of biofilm was quantified by measuring the OD_{595} of crystal violet dissolved in 33% acetic acid. The means and standard deviations of triplicate determinations are presented. Asterix indicate significant differences (*P = 0.05, two-tailed students T-test).

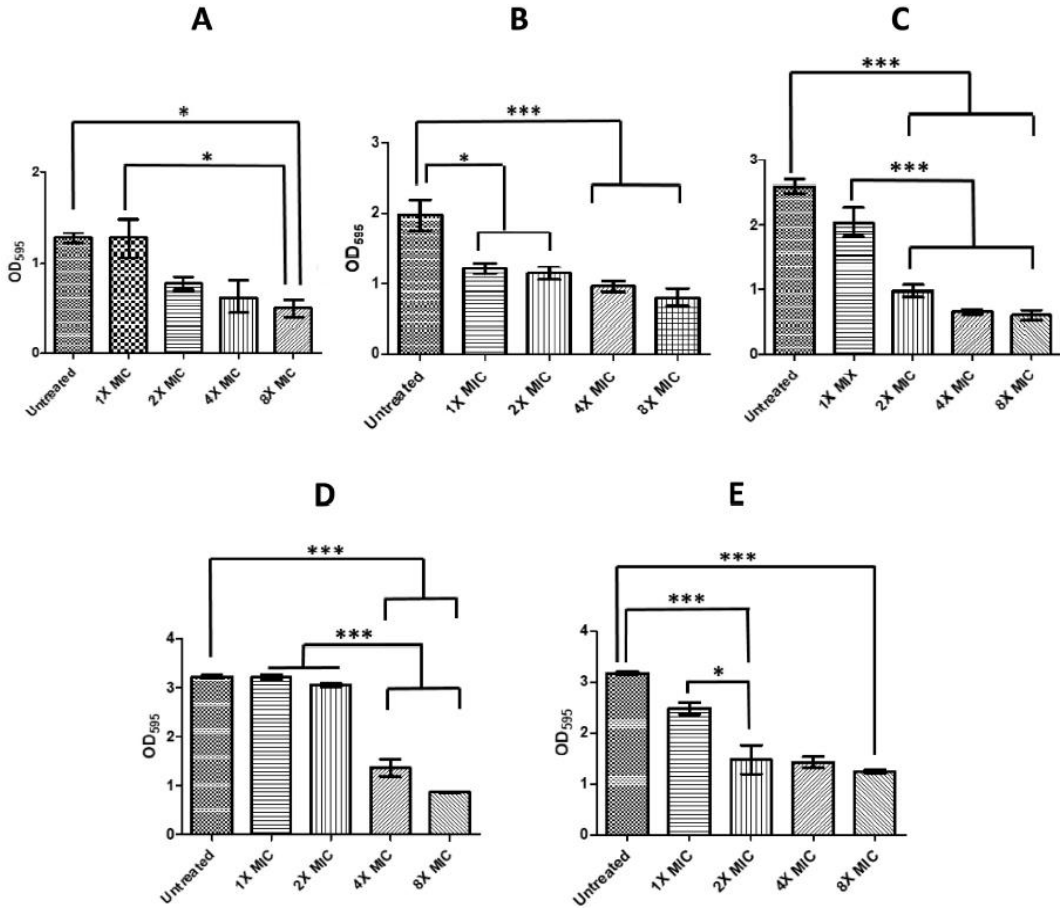

Figure 15. Treatment of established *L. monocytogenes* biofilms with capidermicin. Treatment of established biofilm of *L. monocytogenes* isolates (A) EGDe, (B) F2365, (C) CD749, (D) Ts45 and (E) OB001102 with 1X, 2X, 4X and 8X MIC capidermicin peptide for 24 hours following biofilm formation. The amount of biofilm present was quantified following staining and measuring the OD_{595} of 0.05% crystal violet dissolved in 33% acetic acid. The mean and standard deviations of triplicate determinations are presented. Asterix indicate significant differences (*P = 0.05, ***P = 0.001; two-tailed students T-test).

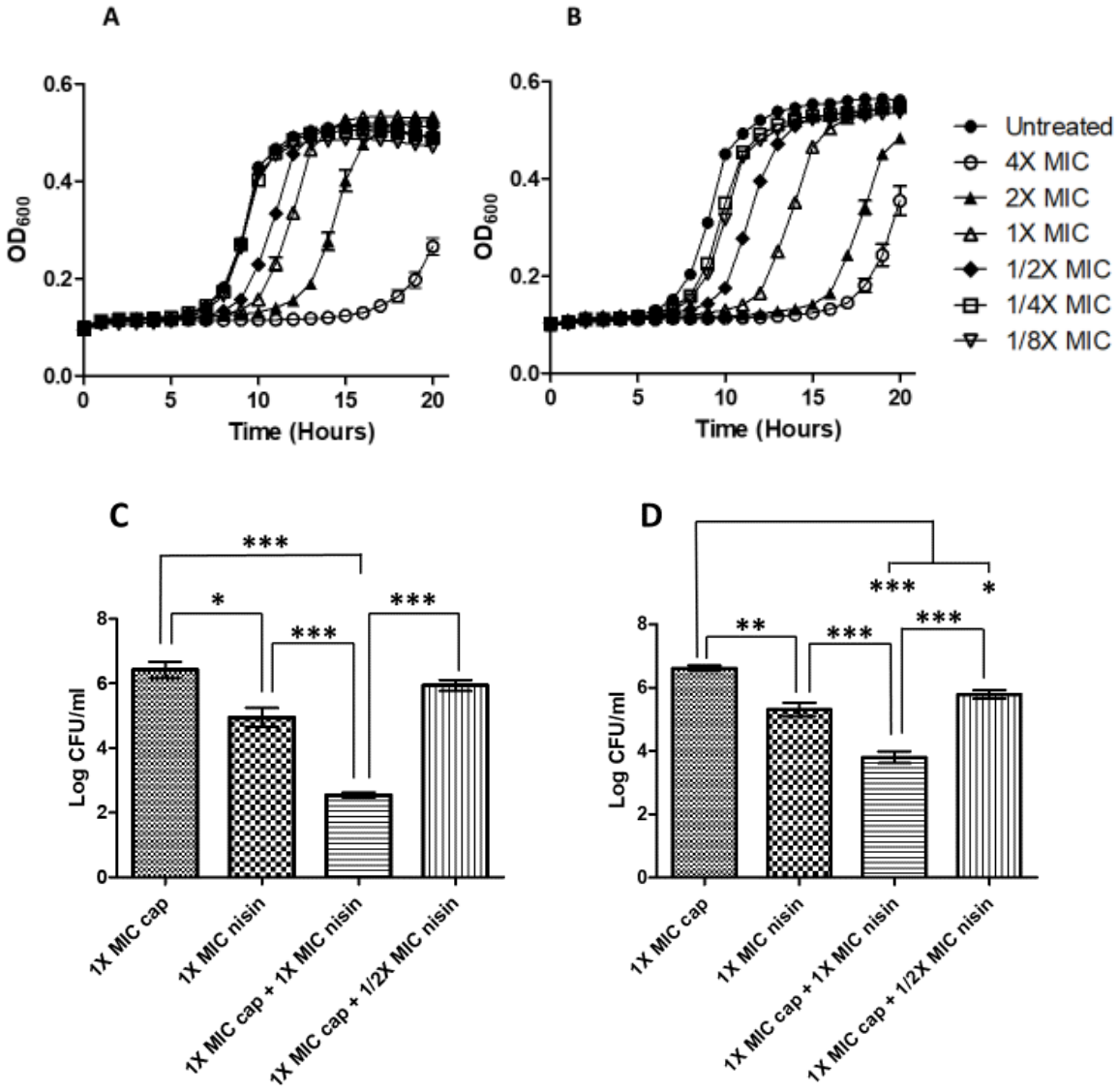

Figure 16. Growth curve analysis of increasing concentrations of capidermicin against (A) *L. monocytogenes* EGDe and (B) F2365 with a starting inoculum of 10^5 CFU/ml. Growth was measured via triplicate readings at OD_{600} over a period of 20 hours. For time-point peptide combination assays against strains (C) *L. monocytogenes* EGDe and (D) F2365 with a starting inoculum of 10^5 CFU/ml, upon exposure to capidermicin alone (1X MIC) and in combination with nisin A (1X MIC or 1/2X MIC nisin) in BHI broth for a period of 3 h at 37°C. All assays were carried out in triplicate. The mean and standard

deviations of triplicate determinations are presented. Asterix indicate significant differences (*P = 0.05, **P = 0.005, *** P = 0.001; two-tailed students T-test).

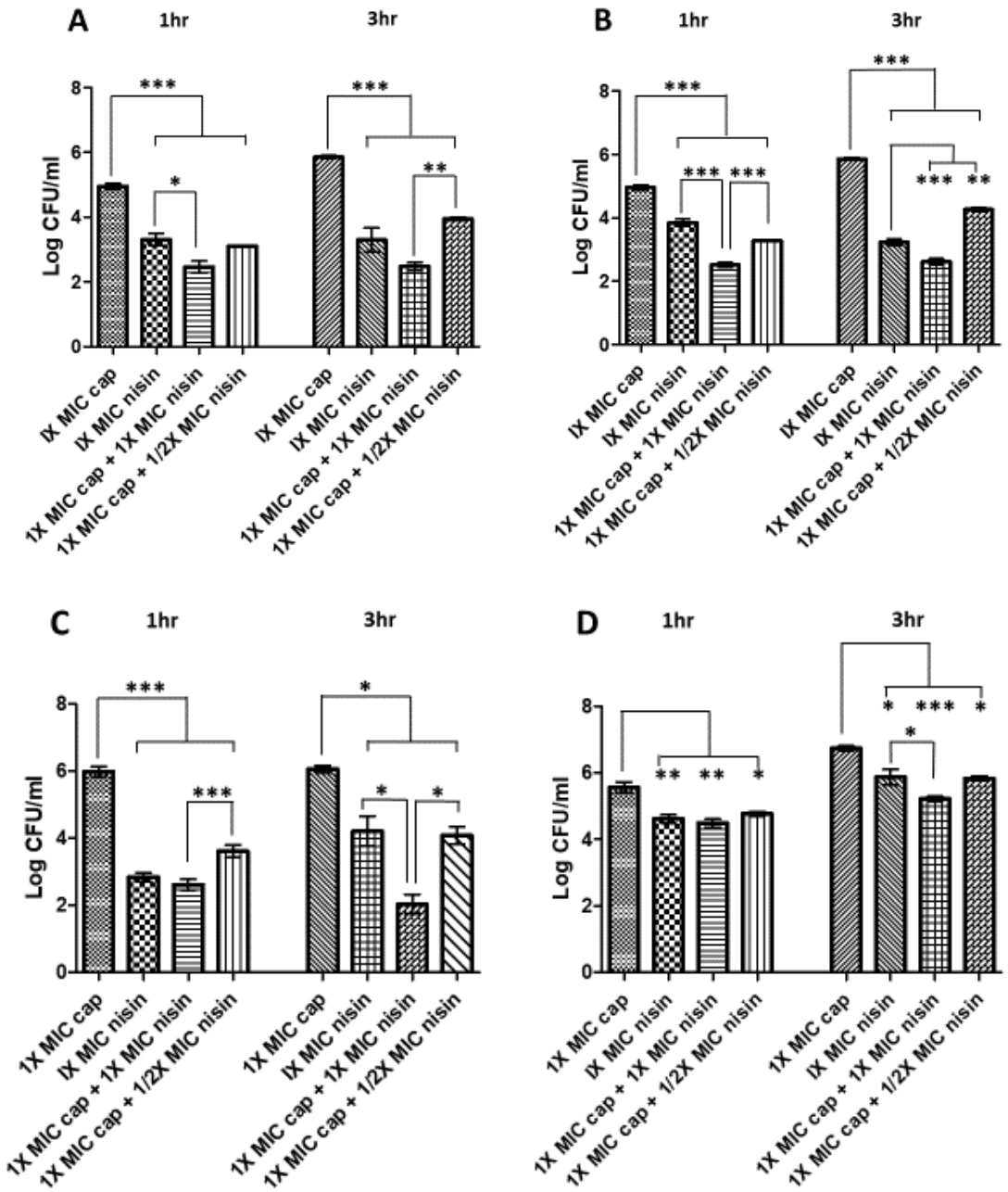

Figure 17. Model Food analysis. The impact of capidermicin alone, and in combination with nisin A peptide, against *L. monocytogenes* EGDe (starting inoculum of 10^5 CFU/ml) in a commercially available (A) cottage cheese and (B) chocolate milk at 22°C as determined by CFU counts after 1-hour and 3-hour exposure periods. Capidermicin alone, and in combination with nisin A peptide against *L. monocytogenes* F2365 (C) cottage cheese and (D) chocolate milk Data points are the average of triplicate experiments.

Asterix indicates significant differences (*P = 0.05, ***P = 0.001; two-tailed students T-test).

Chapter 4

Bioengineering and rational design of the antimicrobial peptide nisin leads to the identification of variants with enhanced specific activity and protease resistance to clinically relevant pathogens

David Lynch, Paula O' Connor, Colin Hill, Máire Begley and Des Field

4.1 Abstract

The original and most extensively studied lantibiotic nisin A is a broad spectrum bacteriocin that inhibits Gram-positive bacteria including problematic food-pathogens and clinically relevant antibiotic resistant bacteria. Nisin is gene-encoded and is amenable to peptide bioengineering, which can result in novel derivatives that can be screened for more desirable properties e.g. altered bioactivity. In the present study, a bank of 48 nisin derivatives with mutations in the hinge region (naturally asparagine N20, methionine M21 and lysine K22) were screened for their ability to inhibit indicator bacteria that possess the nisin resistance protein (NSR) i.e. NSR+ strains namely *Lactococcus lactis* MG1614 (NSR-) and *Lactococcus lactis* MG1614 pNP40 (NSR+) using an agar-based deferred antagonism assay. The largest zones of inhibition were observed for three mutants - N20P, M21V and K22T. Subsequent experiments focused on using a "rational design" approach where these three individual hinge mutations were made in a strain producing a protease resistant version of nisin i.e. nisin PV. Peptides were purified from the resultant mutants N-PV, M-PV and T-PV. Enhanced bioactivity against problematic NSR+ bacteria and protease resistance was confirmed. This is the first study to generate nisin mutants with combined enhanced specific activity in addition to improved resistance to proteolytic cleavage by NSR. These nisin derivatives with enhanced resistance and bioactivity to specific NSR resistance mechanisms could prove useful in the fight against clinical pathogens.

4.2 Introduction

The rise and emergence of multi-resistant pathogenic bacteria coupled with an endlessly reducing repertoire of effective antimicrobials poses a severe threat to human health across the world. For the past 60 years, pharmaceutical companies have been trying to fight the onslaught of antibiotic resistance but with little success. Therefore, the need to explore alternative antimicrobial compounds that can overcome resistance mechanisms, remains paramount. Over the past couple of decades, one large group of antimicrobial peptides, the bacteriocins, has received interest and in particular the lantibiotic subgroup because of their high potency, low toxicity and amenability to bioengineering (Bierbaum and Sahl, 2009; Field et al., 2019, 2015a; Rouse et al., 2012). The term lantibiotic or "lanthionine-containing antibiotic" is derived from the presence of unusual amino acids including lanthionine and ring structures within the peptides (Bierbaum and Sahl, 2009). Lantibiotics are produced by a wide range of Gram-positive bacteria, and have demonstrated exceptional antimicrobial activity against numerous problematic pathogens (Field et al., 2015c, 2012). The original, and most intensively studied lantibiotic is nisin, and is the only peptide to have received US Food and Drug Administration (USFDA) approval (Shin et al., 2016). This polycyclic, 34 amino acid peptide uses the peptidoglycan precursor lipid II as a docking molecule, a step that facilitates two bactericidal activities, the inhibition of peptidoglycan biosynthesis and membrane pore formation (Breukink et al., 1999; Field et al., 2019; Wiedemann et al., 2001b).

In 1992, Kuipers and colleagues (Kuipers et al., 1992) commenced the *in vivo* engineering of the nisin peptide, generating a number of valuable derivatives. Over the last decade however, additional nisin derivatives have been generated with enhanced antimicrobial activity against pathogenic bacteria (Field et al., 2019, 2015c, 2012; Rouse

et al., 2012), with the majority of these mutations located within the 'hinge' region of the peptide i.e. asparagine N20, methionine M21 and lysine K22 (Figure 6; Figure 18). Indeed, the importance of the hinge has been highlighted on several occasions (Field et al., 2008; Healy et al., 2013; Schmitt et al., 2019; Yuan et al., 2004; Zhou et al., 2015). This 3 amino acid long region has been implicated in playing an important role during insertion of the C-terminus of nisin into the membrane, by linking the two functional domains and providing conformational flexibility to the peptide (Hasper et al., 2004). Additionally, the hinge region plays an important role in the recognition of the immunity protein *Nis*I (Zaschke-Kriesche et al., 2019).

Studies have revealed that the bactericidal efficacy of nisin has been compromised by the occurrence of a nisin resistance protein (NSR) produced by some *Lactococcus* and *Streptococcus* strains, which provides resistance through the proteolytic cleavage of nisin (Field et al., 2019; Froseth and McKay, 1991). The ability of NSR to cleave the peptide bond between MeLan28 and Ser29 (Figure 18) results in a truncated nisin peptide ($nisin^{1-28}$), that exhibits a reduced affinity for the bacterial membrane, and up to a 100-fold decrease in bactericidal activity (Sun et al., 2009). Recently a PCR-based bioengineering strategy was employed to target the DNA encoding the serine 29 and isoleucine 30 residues of nisin A, resulting in the identification of nisin PV (S29P-I30V). Nisin PV displayed a 20-fold increase in bioactivity against NSR^{+} strains when compared with wild type nisin A and revealed that nisin derivatives could be created which possessed improved resistance to proteolytic cleavage, whilst retaining full antimicrobial activity.

Given the merits of manipulating the nisin peptide, and how peptide functionality can be modified significantly by changing as little as one residue (Field et al., 2008; Islam et al., 2009), here our attention turns to 'hinge region'. In this study, we applied the

approach of site-specific mutagenesis techniques to create nisin 'hinge region' derivatives which possess enhanced resistance to proteolytic cleavage by NSR whilst also maintaining antimicrobial activity. This involved screening a bank of hinge derivatives against NSR^{+} strains to identify mutants with enhanced activity. This process led to the identification of three hinge derivatives that were invulnerable to NSR. This study sought to go a step further through the site-specific mutagenesis of all three hinge residues of the nisin PV peptide with the aim of generating nisin mutants with combined enhanced specific activity in addition to improved resistance to proteolytic cleavage by NSR. This discovery highlights the benefits of bioengineering nisin with a view to generating derivatives with superior bioactivity against clinically relevant *Streptococcus* and *Staphylococcus* NSR+ pathogenic strains.

4.3 Material and Methods

4.3.1 Bacterial strains and growth conditions

The strains used in this study are listed in Table 6. *L. lactis* strains were grown in M17 broth supplemented with 0.5% glucose (GM17) or GM17 agar at 30°C. *E. coli* was grown in Luria-Bertani (LB) broth with vigorous shaking or agar at 37°C. *Staphylococcus* and *Streptococcus* strains were grown in Brain Heart Infusion (BHI) or BHI agar and Tryptic Soy Broth (TSB) supplemented with 0.6% Yeast Extract (TSB-YE) at 37°C, respectively. Antibiotics were used where indicated at the following concentrations: Chloramphenicol (Cm) at 10 and 20 μg ml–1 respectively for *L. lactis* and *E. coli*.

4.3.2 Creation of truncated hinge derivatives (N20P$^{1-28}$, M21V$^{1-28}$ and K22T$^{1-28}$)

Mutagenesis of the *nis*A gene was carried out as described previously (Field et al., 2019). Briefly, saturation mutagenesis was carried out using pDF22 (pCI372-*nis*A$^{1-28}$) as a template. For N20P$^{1-28}$, M21V$^{1-28}$ and K22T$^{1-28}$, the following oligonucleotides were used; N20P$^{1-28}$ FOR and N20P$^{1-28}$ REV, M21V$^{1-28}$ FOR and M21V$^{1-28}$ REV, K22T1-28-FOR and K22T1-28-REV (Table 7), all containing a TAA stop codon in position of the native AGT codon (serine 29). PCR amplification was performed in a 50 μl reaction containing approximately 0.5 ng of target DNA (pDF22), 1 unit Phusion High-Fidelity DNA polymerase (Finnzymes, Finland), 1 mM dNTPs and 500 ng each of the appropriate forward and reverse oligonucleotide. 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 (Invitrogen, Dublin, Ireland), plasmid DNA was isolated and sequenced using primers pCI372FOR and pCI372REV (Table 7) to verify that the correct mutation had had been

introduced in each case. The purified plasmid products were subsequently introduced by electroporation into the strain *L. lactis* NZ9800.

4.3.3 Site-directed mutagenesis

Site-directed mutagenesis of the *nis*PV gene was carried out using a PCR-based mutagenesis strategy using pDF05-PV (Field et al., 2019) as template and the relevant primers in each case. For combination mutants N-PV, M-PV and T-PV, the following oligonucleotides were used; N20P-PV FOR and N20P-PV REV, M21V-PV FOR and M21V-PV REV, K22T-PV FOR and K22T-PV REV (Table 7). Plasmids from candidates were sequenced using pCI372Rev to verify the deliberate mutation and to confirm no other alternations had been introduced. Plasmid DNA from successful strains was used to transform *L. lactis* NZ9800. Transformants were analysed by MS to verify production of the nisin peptide mutants.

4.3.4 Bioassays for antimicrobial activity

Deferred antagonism assays were performed by replicating producing strains on GM17 agar plates and allowing them to grow overnight before overlaying with either GM17/BHI/TSB-YE agar (0.75% w/v agar) and seeded with the nisin resistance protein-expressing indicator strain *Lactococcus lactis* MG1614 (NSR-)/MG1614 pNP40 (NSR+), *Staphylococcus capitis* (NSR+) or *Streptococcus agalactiae* (NSR+). For higher throughput screening of the nisin producing variant banks, deferred antagonism assays were performed by replicating strains using a 96 pin replicator (Boekel) or spotting 5 µl of a fresh overnight culture on GM17 agar plates and allowing them to grow overnight. Following overnight growth, the strains were subjected to UV radiation (High performance UV transmitter, Upland, Ca, USA) for 30 min prior to overlaying with either GM17/BHI/TSB-YE agar (0.75% w/v agar) seeded with the appropriate indicator. Zones

of inhibition were measured with digital callipers and calculated as the diameter of the zone of clearing minus the diameter of the producing colony.

4.3.5 Nisin purification

Purification of nisin from *Lactococcus lactis* NZ9700 (nisin A producer) or the nisin mutant of interest was performed as described previously by (Field et al., 2019). Two litres of modified Tryptone Yeast (TY) broth were inoculated with the culture at 0.5% and incubated at 30°C overnight. The culture was centrifuged at 7,000 rpm for 20 min. The cell-free supernatant was removed, collected, and passed through 60g of pre-equilibrated Amberlite XAD16N beads (Sigma Aldrich). The beads were washed with 30% ethanol and eluted with 400ml 70% isopropanol (IPA) + 0.1% trifluoroacetic acid (TFA). The cell supernatant was further concentrated through rotary-evaporation to approximately 250ml. The cell pellet was resuspended in 250 ml of 70% isopropanol (IPA) 0.1% trifluoroacetic acid (TFA) and stirred at room temperature for approximately 3 hours. The cell debris was removed by centrifugation at 6500 g for 15 min and the supernatant retained. The supernatant and cell pellet elution's (approximately 650mls) were pooled and the 70% isopropanol (IPA) 0.1% trifluoroacetic acid (TFA) was evaporated using a rotary evaporator (Buchi) and the sample pH adjusted to 4, before applying to a 10 g (60 ml) Varian C-18 Bond Elution Column (Varian, Harbor City, CA) pre-equilibrated with methanol and water. The columns were washed with 60mls of 30% ethanol and the inhibitory activity was eluted in 60ml of 70% IPA + 0.1% TFA. About 12 ml aliquots were concentrated to 2 ml through the removal of propan-2-ol by rotary evaporation. 2ml aliquots were applied to a Phenomenex (Phenomenex, Cheshire, UK) C12 reverse phase (RP)-HPLC column (Jupiter 4u proteo 90 Å, 250 × 10.0 mm, 4 μm) previously equilibrated with 25% acetonitrile – 0.1% TFA. The column was subsequently

developed in a gradient of 25 % acetonitrile – 0.1% TFA to 50% acetonitrile containing 0.1% TFA from 10 to 40 min at a flow rate of 3.2 ml min–1. The relevant active fractions were collected and pooled, subjected to rotary-evaporation to remove the acetonitrile and freeze-dried. Bacteriocin activity was monitored throughout the purification procedure by well diffusion assay using a sensitive indicator strain *Lactococcus lactis* MG1614.

4.3.6 Mass spectrometry analysis

For colony mass spectrometry (CMS) experiments, bacterial colonies were collected with sterile plastic loops and mixed with 50 µl of 70% IPA adjusted to pH 2 with HCl. The suspension was vortexed, the cells centrifuged in a benchtop centrifuge at 8260 g for 2 min and the supernatant was removed for analysis. Mass Spectrometry in all cases was performed with an Axima CFR plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5 µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic 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 (resuspended 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 positive-ion reflectron mode.

4.3.7 Minimum inhibitory concentration (MIC) assays

Minimum inhibitory concentration (MIC) determinations were carried out in triplicate in 96-well microtitre plates. All 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) bovine serum albumin (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, subcultured into fresh broth and allowed to grow to an OD600 of ~0.5, diluted to a final concentration of 10^5 cfu ml^{-1} in a volume of 0.2 ml. Nisin PV and nisin mutant peptides were adjusted to a 30 μM or 5 μM (*Streptococcus*), 7.5 μM or 500 nM (*L. lactis*) starting concentrations and two-fold serial dilutions of each peptide were added to the target strain. After incubation for 16 h at 37°C, the MIC was read as the lowest peptide concentration causing inhibition of visible growth.

4.3.8 Proteolytic activity of NSR against N20P, M21V, K22T and N-PV, M-PV and K-PV combination mutants

To investigate whether NSR could degrade the N20P, M21V, K22T and PV mutants *in vivo*, a series of peptide release assays were performed according to the method described by Stein and colleagues (Stein et al., 2003). Briefly, flasks containing 50 ml of GM17 medium were inoculated with a 1/100 volume of overnight of *L. lactis* subsp. *lactis* biovar diacetylactis DRC3 and incubated at 30°C until the OD600 reached 0.6. Cells were harvested by centrifugation at 4,000 g for 10 min, washed twice with 50 mM Tris-HCl (pH 6.0), centrifuged again and resuspended in incubation buffer (50 mM sodium phosphate buffer [pH 6.0], 1 M NaCl and 1% [wt/vol] glucose) at 1 ml aliquots in microcentrifuge tubes. Nisin A, N20P, M21V, K22T, PV and nisin combination mutants (50 μg) was added, and the aliquots were incubated for 30 min at 30°C with gentle shaking. After incubation, the aliquots were centrifuged for 10 min at full speed in a micro-centrifuge, and the supernatants collected. The harvested cell pellets were washed with the incubation buffer, gently mixed with 1 ml of 20% acetonitrile in water containing 0.1% TFA and incubated with gentle shaking at 30°C for 5 min. The cells were removed by centrifugation at 12,000 g for 10 min, and the supernatants were collected. The

collected supernatants (500 µl each) were subjected to RP-HPLC and MS analysis as described above.

4.4 Results

4.4.1 Screening of nisin hinge variants against a target strain expressing the nisin resistance protein (NSR^+)

Previous random, site-directed and site- saturation mutagenesis provided a substantial amount of data with respect to the consequences of the alteration of specific residues within the nisin hinge, with the most significant outcome having been the identification of several derivatives with enhanced activity against specific Gram-positive pathogens (Field et al., 2010b, 2008). Despite this, these derivatives had not previously had the benefit of being tested against strains possessing the specific nisin resistance determinant NSR. To that end, the bioactivity of the 15 *L. lactis* NZ9800 pDF05-N20X (Asn 20 altered to Cys, Ala, Ser, Thr, Val, Leu, Ile, Pro, Phe, Tyr, Trp, Asp, Arg or His), 18 *L. lactis* pDF05-M21X (Met 21 altered to Gln, Cys, Gly, Ala, Ser, Thr, Val, Leu, Ile, Pro, Phe, Tyr, Trp, Arg or Lys) and 15 *L. lactis* pDF05-K22X (Lys 22 altered to Gln, Gly, Ala, Ser, Thr, Val, Leu, Pro, Met. Phe, Trp, Asp, Glu, Arg, His or Lys) derivatives (Field et al., 2008) were tested against indicator strains *L. lactis* MG1614 pNP40 (NSR^+) and *L. lactis* MG1614 (NSR^-) as negative control by deferred antagonism assays, with zones of inhibition being assessed relative to that of nisin A producer and nisin PV (Figure 19).

Against the *L. lactis* strain MG1614, the nisin variant bioactivities observed were in agreement with those of Field and co-workers (Field et al., 2008) whereby derivatives N20P, M21V, M21A, K22T and K22S exhibited the greatest zones of inhibition (Table 8). Surprisingly, the activity of these variants also appeared enhanced against the NSR+ *L. lactis* MG1614 pNP40 when compared to the wild type nisin A peptide, though the zones of inhibition were somewhat reduced compared to those produced against the strain lacking the protease (Table 8; Figure 19). Accordingly, the N20P, M21V and K22T derivatives were selected as representing the most beneficial mutations. The nisin N20P,

M21V and K22T were found to possess the greatest bioactivity of all hinge region variants against the NSR+ *L. lactis* MG1614 pNP40, and they were selected for further analysis (Figure 19).

4.4.2 Specific activity of nisin hinge derivatives

Additional deferred antagonism assays with 23 clinical Gram-positive isolates expressing NSR (13 *Staphylococcus capitis* and 10 *Streptococcus agalactiae*) were carried out against the hinge mutants of interest (Table 6; Figure 20 and 21). Against the clinical *S. capitis* isolates, the nisin variant bioactivities of N20P and M21V exhibited the greatest zones of inhibition (Figure 20). The nisin N20P and M21V producers displayed an ability to inhibit all *S. capitis* isolates, while K22T inhibited eight isolates with significantly smaller zones of inhibition. In contrast, the K22T derivative exhibited the greatest bioactivity against the clinical *S. agalactiae* strains (Figure 21).

The fact that the bioactivity of the hinge region mutants is improved against clinically relevant NSR+ strains, and to ensure that their enhanced activity against NSR+ targets was not simply due to an increase in production, or improved peptide diffusion through the agar matrix, and thus is most likely a consequence of the peptides possessing altered specific activity against NSR+ strains, nisin N20P, M21V and K22T were purified using the Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) protocol routinely employed to purify nisin, to allow determination of their specific activity. The specific activity of the peptides was assessed using broth-based MIC determination assays against *L. lactis* MG1614 (NSR^-), *L. lactis* MG1614 pNP40 (NSR^+), *S. agalactiae* UCC67 (NSR^+), and *S. uberis* ATCC 700407 (NSR^+). In each case, the specific activity was compared to that of nisin PV. MIC assays were carried out using equimolar concentrations of nisin PV and each nisin hinge derivative against the NSR+

bacterial indicators. MIC values for N20P and K22T against *L. lactis* MG1614 (NSR^-) were determined to be 0.125 and 0.125 µg ml^{-1} respectively, which was the same as that observed for nisin PV (Table 9). In contrast, M21V exhibited a two-fold increase in specific activity (0.065 µg ml^{-1}) compared to the nisin PV peptide. Furthermore, all three hinge derivative peptides (N20P, M21V and K22T) were observed to have increased specific activity against the *L. lactis* MG1614 pNP40 strain when compared to the wild type nisin A (2 µg ml^{-1}) with MICs of 0.5, 0.25 and 0.25 µg ml^{-1} respectively, and in agreement with observations from the deferred antagonism assays (Figure 19). When assessed against *S. uberis* ATCC 700407, a strain shown to possess NSR (Field et al., 2019), M21V alone displayed enhanced activity with an MIC of 1.5 µg ml^{-1} (0.468 µM), while the MIC of nisin PV was 2 µg ml^{-1} (0.625 µM), in agreement with that previously established (Field et al., 2019).

4.4.3 Creation and specific activity determination of truncated nisin hinge variants

Since these initial investigations could not determine whether the enhanced bioactivity associated with the nisin hinge derivatives against the NSR+ strains could be attributed to an intact or cleaved peptide, truncated versions of each derivative i.e. $N20P^{1–28}$, $M21V^{1–28}$, and $K22T^{1–28}$ were generated (through site-specific mutagenesis) for comparative purposes in addition to the nisin $A^{1–28}$ peptide control as described previously (Field et al., 2019). DNA sequencing and mass spectrometry analysis confirmed the appropriate mutation and production of each truncated nisin variant respectively (data not shown). The bioactivity of these truncated mutants was initially investigated through deferred antagonism assays using *L. lactis* MG1614 (NSR-) and *L. lactis* MG1614 pNP40 (NSR+) in addition to the relevant controls: wild type nisin A, and nisin PV. Notably, as was previously observed with nisin A1–28 (Field et al., 2019), the truncated mutants

$N20P^{1–28}$, $M21V^{1–28}$, $K22T^{1–28}$ had lost the ability to induce (data not shown). Consequently, nisin was added to the agar (in the form of nisaplin) to a final concentration of 100ng/ml to stimulate production of each peptide variant. Despite this, bioactivity from the truncated mutants was severely reduced, whereby a zone of inhibition was barely detectable for nisin $A^{1–28}$, $N20P^{1–28}$, $M21V^{1–28}$, $K22T^{1–28}$. This was not due to diminished production since sufficient peptide was readily obtained from purification steps following induction with nisaplin (data not shown).

As expected, the truncated mutants proved to be much less active with MIC values of 3 µg $ml^{–1}$ and 3 µg $ml^{–1}$ for $N20P^{1–28}$ and $M21V^{1–28}$ respectively and 6 µg $ml^{–1}$ in the case of $K22T^{1–28}$. These values were in agreement with the MIC previously established for nisin $A^{1–28}$ (3 µg $ml^{–1}$)(Field et al., 2019). Against *L. lactis* MG1614 pNP40, the $M21V^{1–28}$ and $K22T^{1–28}$ truncated mutants exhibited reduced activity and were in agreement with the MIC previously established for the truncated nisin $A^{1–28}$ peptide (3 µg $ml^{–1}$; 0.937 µM). However, the MIC for $N20P^{1–28}$ was observed to be lower than that of the other truncated derivatives with a MIC of 1.5 µg $ml^{–1}$ (Table 9).

4.4.4 Proteolytic activity of NSR against nisin A and nisin hinge derivatives

To gain further insight into the rationales behind the enhanced specific activity of the hinge derivatives against NSR+ strains as observed from MIC data, the *in vivo* proteolytic activity of NSR was examined with a series of peptide release assays using nisin A, N20P, M21V and K22T as substrates in the presence of *L. lactis* DRC3 (NSR+) cells (Sun et al., 2009). Following incubation and centrifugation, nisin peptides or their degraded fragments in the supernatant were analysed by RP-HPLC and mass spectrometry in a bid to determine the degree of digestion by the NSR enzyme. Two peaks were apparent, one major and one minor peak eluting between 30 and 35 minutes for all nisin derivative

N20P, M21V and K22T (Figure 22B, C and D; respectively). In the case of the wild type nisin A, two peaks were also apparent (Figure 22A), though a slightly lower ratio between peak size was evident. Mass spectrometric analysis of the eluted HPLC fractions revealed the major peak to contain the undigested peptide in each case: Figure 22A (top) intact wild type, (22B top) intact N20P, (22C top) intact M21V and (22D top) intact K22T. The fraction corresponding to the minor peak contained the digested peptide in each case (22A bottom) truncated wild type, (22B bottom) truncated N20P, (22C bottom) truncated M21V and (22D bottom) truncated K22T. Additionally, area under the curve analysis was undertaken in a bid to quantify the relative amount of intact peptide versus truncated peptides for comparison purposes. Analysis revealed that 24.44% of wild type peptide was in the truncated form (Table 10). In contrast, 9.93% of N20P, 18.64% M21V and 16.81% of K22T were shown to have been cleaved by NSR (Table 10).

4.4.5 Generation of nisin combination mutants

It was speculated that the bioactivity of hinge mutants could be further enhanced through the introduction of a selection of changes previously found to enhance activity against NSR+ strains. Thus, a site-specific mutagenesis of all three hinge residues of the nisin PV peptide was carried out using the relevant pDF05-PV plasmid (Table 6) and oligonucleotides (Table 7) as a template to incorporate the favourable N20P, M21V and K22T mutations and transformed into *L. lactis* NZ9800. DNA sequencing and mass spectrometry analysis confirmed the appropriate mutation and production of each nisin variant peptide respectively (data not shown). The bioactivity of these combination mutants (N20P = N-PV, M21V = M-PV, K22T = T-PV) was assessed through deferred antagonism assays using *L. lactis* MG1614 (NSR-) and *L. lactis* MG1614 pNP40 (NSR+). As expected, nisin PV retained full activity when compared to the wild type against the

NSR+ producing strains. Moreover, the N-PV, M-PV and T-PV producers displayed enhanced bioactivity against both the NSR^- and NSR^+ target strains compared to the parental nisin A and were at least equivalent to PV.

The bioactivities of the N-PV, M-PV and T-PV producers were further assessed using the clinical NSR+ pathogens *Streptococcus agalactiae* and *Staphylococcus capitis* isolates as indicators (Table 6). Notably, the bioactivity of T-PV against *S. agalactiae* (Figure 21) was comparable to that of nisin PV. Against NSR+ *S. capitis,* the N-PV mutant exhibited enhanced bioactivity by inhibiting 12/13 neonatal isolates, with nisin PV inhibiting 9/13 (data not shown). These results are consistent with previous studies, with K22T and N20P displaying enhanced activity against *Streptococcus* and *Staphylococcus*, respectively. Furthermore, N-PV exhibited enhanced bioactivity when compared to that of N20P against the *S. capitis* strains (Figure 20).

As a consequence of the greater bioactivity observed from deferred antagonism assays, nisin combination mutants (N-PV, M-PV and T-PV) were purified. To ensure that the enhanced activity observed for N-PV, M-PV and T-PV was not as a consequence of altered peptide production levels or other potential effects including physico-chemical properties such as improved agar diffusion, the specific activity of the peptides was assessed using broth-based MIC determination assays against *L. lactis* MG1614 (NSR^-), *L. lactis* MG1614 pNP40 (NSR^+), *S. agalactiae* UCC67 (NSR^+), and *S. uberis* ATCC 700407 (NSR^+). In each case, the specific activity was compared to that of nisin PV. MIC assays were carried out using equimolar concentrations of nisin PV and each nisin derivative against the bacterial indicators. When the combination mutants were assessed, the combination of N20P and PV (N-PV) yielded an MIC of 0.0612 μg ml^{-1} compared to 0.125 μg ml^{-1} for PV, corresponding to a 2-fold increase in specific activity against the

NSR+ strain. MIC values of 0.125 µg ml^{-1} and 0.125 µg ml^{-1} were observed for M-PV and T-PV respectively which was equal to that of the nisin PV peptide (Table 9). When assessed against *S. uberis* ATCC 700407, a strain shown to possess NSR (Field et al., 2019), the MIC of nisin PV was 2 µg ml^{-1} (0.625 µM), in agreement with that previously established (Field et al., 2019). Moreover, when the combination mutant M-PV was assessed against *S. uberis* ATCC 700407, an MIC of 1.5 µg ml^{-1} (0.468 µM) was recorded (Table 9) resulting in a 25% increase in specific activity. Against *S. agalactiae* 67, just one derivative proved to be more effective than PV. Here the N-PV variant proved to be twice as potent with an MIC of 1.5 µg ml^{-1} compared to 3 µg ml^{-1}obtained for the nisin PV peptide.

4.5 Discussion

Without question, antibiotic resistance poses one of the greatest health threats of our time (Hawken and Snitkin, 2019). The improper use and overuse of antibiotics has accelerated the evolutionary selection process, which has led to resistance against essentially all approved antibiotics. Thus, there is an urgent need for antimicrobial compounds that can be used as alternatives to, or that can be used in conjunction with conventional antibiotics. In this regard, the lantibiotics, a class of antimicrobial peptides produced by bacteria, are attractive candidates due to their high potency against a wide range of Gram-positive human pathogenic bacteria. Because lantibiotics exert their activity through multiple modes of action, little bacterial resistance to their action has been observed over several decades. Crucially, several operons have been described that encode a protein defence mechanism against lantibiotics including nisin (Khosa et al., 2013). Furthermore, studies have revealed that many human-associated pathogenic bacteria including *S. agalactiae, S. capitis, S. hyicus, S. epidermidis* and *E. faecium* exhibit an innate resistance that has been attributed to the nisin resistance protein (NSR), a membrane-associated protease. This resistance mechanism involves the enzymatic cleavage, and subsequent inactivation of nisin by the removal of the last six residues (Sun et al., 2009). The resulting truncated nisin fragment ($nisin^{1-28}$) displays up to 100-fold lower antibacterial activity and a reduced affinity towards cellular membranes. These lantibiotic resistance mechanisms have the potential to significantly restrict the use of lantibiotics such as nisin as therapeutics. Consequently, novel strategies are required to offset the potential risk of lantibiotic resistance.

Recently, a study involving the bioengineering of nisin gave rise to a derivative which proved to be resistant to proteolytic cleavage by NSR through the systematic

alteration of the cut site (Field et al., 2019). Notably the exchange of serine at position 29 to proline (S29P) resulted in the inability of the nisin resistance protein to cleave nisin (Figure 18).

Indeed, bioengineering and the use of synthetic biology-based (*in vitro* engineering) approaches have been critical for progressing our understanding of bacteriocin activity and structure–function relationships and also in the design of novel peptides with enhanced functionalities with the ultimate aim of making them more attractive from a clinical perspective. A perfect example of this relates to genetic manipulation of the nisin hinge region which has been studied intensively (Field et al., 2008; Healy et al., 2013; Lubelski et al., 2008; Ross and Vederas, 2011; Zhou et al., 2015). Single amino acid changes within the hinge region has generated peptide derivatives with enhanced physico-chemical properties including specific activity and solubility, e.g., N20(P,K), M21(V,K,G, A), K22(T,S) (Field et al., 2008; Yuan et al., 2004, Smith et al., 2016). Moreover, the hinge combinations of AAK, NAI, SLS, AAA and SAA have been shown to retain high antimicrobial activity (Healy et al., 2013). Furthermore, a study by Zhou and colleagues (Zhou et al., 2015) revealed that elongating the hinge region can potentially enhance the activity of peptides against specific bacteria including Gram negative *E. coli.* However, a more recent study by Zachse-Kriesche and co-workers (Zaschke-Kriesche et al., 2019) established that an extended hinge region derivative NMK-IV appeared to be a less suitable substrate for the nisin resistance protein, pointing to the hinge region as being critical with regards to structural recognition by NSR. This prompted us to investigate our complete collection of strains producing nisin variants with the majority of all possible residue alternatives at position 20, 21 and 22 as previously described (Field et al., 2008). Indeed, our initial screen highlighted the

marked enhancement in bioactivity of several hinge mutants but most notably N20P, M21V and K22T against *L. lactis* MG1614 pNP40 (NSR^+). Surprisingly, in MIC assays involving purified peptides, the N20P, M21V and K22T derivatives displayed a 4-8 fold activity over and above that of nisin A, an unexpected observation given that cleavage of the peptide bond between MeLan28 and serine29 would be expected to occur. This prompted a "rational design" approach where these three individual hinge mutations were introduced into a strain that was bioengineered to produce a protease insensitive version of nisin i.e. nisin PV in a bid to enhance the specific activity of this protease resistant peptide. An assessment of the bioactivity of the newly created nisin N-PV, M-PV and T-PV derivatives by deferred antagonism assays revealed they displayed superior bioactivity against a lactococcal NSR+ strain. Further evaluation of N-PV revealed a 2-fold increase in specific activity against *L. lactis* MG1614 pNP40 (NSR^+) and *S. agalactiae* UCC67 when compared to nisin PV. Additionally, the M-PV variant exhibited a 2-fold increase in activity compared to the nisin PV control against *S. uberis* ATCC700407 but not against any of the other NSR+ strains utilised.

To the best of our knowledge, this is the first study to implement the use of site-specific mutagenesis in combination with rational design to generate nisin derivatives possessing both proteolytic resistance to NSR, in addition to enhanced antimicrobial activity. Indeed, the study also reinforces the importance of the hinge region with respect to structure function relationships and reiterates its recent description as a pharmaceutical hotspot (Medeiros-Silva et al., 2019). Although peptide release assays were able to confirm the ability of NSR to proteolytically cleave the hinge derivatives N20P, M21V and K22T, subsequent HPLC analysis revealed that less truncated peptide product was present compared to wild type, suggesting that NSR may be less efficient in processing

the derivative peptides. This, in addition to the enhanced activity of N-PV and M-PV implies the hinge region of nisin represents a new focal point to generate novel nisin derivatives that circumvent nisin resistance mechanisms.

Importantly, bioactivity-based studies have highlighted the enhanced bioactivity of producers N20P, K22T, N-PV, M-PV and T-PV against pathogens associated with neonatal disease (Hayes et al., 2019; Laurent and Butin, 2019). *S. capitis* and *S. agalactiae* remain the leading cause of invasive neonatal disease worldwide and has become increasingly more prevalent amongst non-pregnant adults (Laurent and Butin, 2019). Resistance to second-line antibiotics continues to rise, and worryingly, *S. capitis* have been found to harbour a multidrug resistance profile (including resistance to the usual first-line antibiotics used in neonatal intensive care units (Laurent and Butin, 2019; Simões et al., 2016). Thus, there is a continuing need to monitor resistance and to identify alternative and novel treatments against these opportunistic pathogens.

The global threat of antimicrobial resistance remains a significant problem for the treatment of bacterial infections, particularly those caused by multi-resistant pathogens (Hawken and Snitkin, 2019). The emergence of antimicrobial resistance has surpassed the rate of discovery and development of new and effective antibiotics, resulting in a public-health emergency (Norrby et al., 2005). It has frequently been suggested that nisin could have tremendous value in clinical settings as a result of its high potency against human pathogens (Cavera et al., 2015). However, the future of such antimicrobials necessitates continued studies to investigate the bioavailability of nisin and its derivatives *in vitro* and *in vivo* (Gough et al., 2018). Indeed, if nisin is to expand its therapeutic applications, continuous monitoring of the nisin resistance development in pathogens will need to be undertaken. Additionally, a more in-depth understanding of the NSR

enzymatic resistance mechanisms will provide key information on overcoming bacterial protein defence systems. Improving the bioactivity of nisin can be difficult, because it is already evolutionary optimized against its natural bacterial targets. However, the generation of nisin peptides with value-added properties such as the nisin N-PV and M-PV derivatives described in this study reinforces our belief that bioengineering strategies can be successfully employed to overcome the many challenges associated with peptide antimicrobials including antimicrobial activity, heat stability, solubility, diffusion, and protease sensitivity.

Future work will focus on nisin variants with enhanced antimicrobial activity, such as the newly bioengineered combination mutants N-PV, M-PV and T-PV, for use against clinically relevant pathogens. From a clinical perspective, N20P, K22T, N-PV and T-PV appear to have potential with respect to the treatment of *S. capitis* and *S. agalactiae* which are responsible for neonatal disease. Furthermore, the improved specific activity of N-PV against the causative agent of nosocomial late-onset sepsis in neonatal intensive care units, makes it a promising candidate for the novel treatment of *S. capitis* infections. The improved specific bioactivity of N-PV towards antibiotic resistant *S. capitis* strains is noteworthy and suggests that N-PV merits further investigation with respect to its application against such targets.

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Table 6. Strains and plasmids utilised in this study

Strains/plasmids	Relevant characteristics	Reference
L. lactis NZ9700	Wild type nisin producer	Kuipers et al, 1998
L. lactis NZ9800	*L. lactis* NZ9700Δ*nis*A	Kuipers et al, 1998
L. lactis NZ9800pDF05	*L. lactis* NZ9800 harboring pCI372 with *nis*A under its own promoter	Field et al, 2008
L. lactis NZ9800pDF22	*L. lactis* NZ9800 harboring pCI372-nisA$^{1-28}$	Field et al, 2019
L. lactis NZ9800pDF23	*L. lactis* NZ9800 harboring pCI372-nisA S29P-I30V (PV)	Field et al, 2019
L. lactis NZ9800pDL01	*L. lactis* NZ9800 harboring pCI372-nisA N20P $^{1-28}$	This study
L. lactis NZ9800pDL02	*L. lactis* NZ9800 harboring pCI372-nisA M21V $^{1-28}$	
L. lactis NZ9800pDL03	*L. lactis* NZ9800 harboring pCI372-nisA K22T $^{1-28}$	
L. lactis NZ9800pDL04	*L. lactis* NZ9800 harboring pCI372-nisA M21V-S29P-I30V (MPV)	
L. lactis NZ9800pDL05	*L. lactis* NZ9800 harboring pCI372-nisA K22T-S29P-I30V (TPV)	
L. lactis NZ9800pDL06	*L. lactis* NZ9800 harboring pCI372-nisA N20P-S29P-I30V (NPV)	
E. coli Top10	Intermediate cloning host	Invitrogen
Indicator organisms		
L. lactis subsp. *lactis* biovar diacetylactis DRC3	Carries the nisin-resistance gene *nsr*	(Field et al., 2019)
L. lactis MG1614	Nisin sensitive indicator. Rifampicin- and streptomycin-resistant derivative of MG1363	(Gasson, 1983)
L. lactis MG1614 pNP40	*L. lactis* strain that carries the nisin-resistance gene *nsr* on the plasmid pNP40	O'Driscoll et al, 2006
Staphylococcus capitis AY41		(Simões et al., 2016)
Staphylococcus capitis BA06		

Strains/plasmids	Relevant characteristics	Reference
Staphylococcus capitis AR18	Neonatal isolates, carries the nisin-resistance gene *nsr*	
Staphylococcus capitis AD69		
Staphylococcus capitis AV70		
Staphylococcus capitis AV80		
Staphylococcus capitis BH68		
Staphylococcus capitis AL01		
Staphylococcus capitis BD67	Neonatal isolates, carries the nisin-resistance gene *nsr*	
Staphylococcus capitis AD43		
Staphylococcus capitis BI77		
Staphylococcus capitis BA01		
Staphylococcus capitis BB27		
Streptococcus agalactiae UCC67	Neonatal isolates, carries the nisin-resistance gene *nsr*	(Hayes et al., 2019)
Streptococcus agalactiae UCC 85		
Streptococcus agalactiae UCC 87		
Streptococcus agalactiae UCC 223		
Streptococcus agalactiae UCC 239		
Streptococcus agalactiae UCC273		
Streptococcus agalactiae UCC 364		
Streptococcus agalactiae UCC 395		
Streptococcus agalactiae UCC TS-7		
Streptococcus agalactiae UCC TS-9		
Streptococcus uberis ATCC 700407	Carries the nisin-resistance gene *nsr*	ATCC culture strain

Table 7. Oligonucleotides utilised in this study

Primer name	Sequence 5' – 3'
N20P$^{1-28}$ FOR	5' ATGGGTTGTCCCATGAAAACAGCAACTTGTCATTGT
N20P$^{1-28}$ REV	5' TGTTTTCATGGGACAACCCATCAGAGCTCCTGTTTTACA
M21V$^{1-28}$ FOR	5' GTTGTAACGTGAAAACAGCAACTTGTCATTGTTAAATT
M21V$^{1-28}$ REV	5' GCTGTTTTCACGTTACAACCCATCAGAGCTCCTGTTTT
K22T$^{1-28}$ FOR	5' TGTAACATGACAACAGCAACTTGTCATTGTTAAATTCAC
K22T$^{1-28}$ REV	5' AGTTGCTGTTGTCATGTTACAACCCATCAGAGCTCCTGT
N20P-PV FOR	5' ATGGGTTGTCCTATGAAAACAGCAACTTGTCATTGTCCT
N20P-PV REV	5' TGTTTTCATAGGACAACCCATCAGAGCTCCTGTTTTACA
M21V-PV FOR	5' 5' GTTGTAAC*G*TTAAAACAGCAACTTGTCATTGT 3'
M21V-PV REV	5' GCTGTTTTAACGTTACAACCCATCAGAGCTCCTGT 3'
K22T-PV FOR	5' GTAACATGACTACAGCAACTTGTCATTGT 3'
K22T-PV REV	5' GTTGCTGTAGTCATGTTACAACCCATCAGAGCTCC 3'
pCI372FOR	5'- CGGGAAGCTAGAGTAAGTAG -3'
pCI372REV	5'- ACCTCTCGGTTATGAGTTAG -3'

Table 8. Bioactivity of nisin derivatives at position 20 (N20X), 21 (M21X) and 22 (K22X) against *L. lactis* MG1614 (NSR-) and *L. lactis* MG1614 pNP40 (NSR+). Values are the average of duplicate deferred antagonism assays and represent zone size [diameter of zone] relative to that of the NZ9800 pCI372-nisA (WT) control. Bold font denotes activity greater than WT = wild type

N20X	MG1614 (mm)	MG1614 pNP40 (mm)	M21X	MG1614 (mm)	MG1614 pNP40 (mm)	K22X	MG1614 (mm)	MG1614 pNP40 (mm)
C	17.48	6.50	**G**	11.99	5.14	**F**	10.09	6.81
D	11.46	4.20	**Q**	**17.98**	**12.09**	**D**	0	0
T	10.23	6.68	**N**	11.65	4.49	**V**	**14.89**	**11.37**
V	11.89	8.03	**S**	9.16	4.11	**W**	10.54	8.48
P	**15.15**	**11.08**	**A**	**18.81**	**13.75**	**T**	**19.92**	**13.48**
L	10.86	9.53	**C**	7.62	2.07	**M**	**14.33**	**9.43**
I	9.58	7.53	**I**	15.11	10.95	**L**	**15.28**	**10.11**
H	11.21	8.39	**L**	11.93	7.46	**E**	**15.60**	**11.39**
R	7.73	5.80	**T**	16.50	10.92	**S**	**17.97**	**11.50**
Y	10.48	7.10	**WT**	13.89	8.88	**Q**	**14.09**	**8.92**
WT	13.67	8.95	**V**	**18.53**	**13.25**	**R**	10.24	5.96
S	12.95	9.13	**P**	8.15	1.95	**WT**	13.52	8.88
W	6.42	4.65	**W**	6.14	2.46	**A**	**16.77**	**11.88**
F	7.49	6.45	**Y**	13.73	8.92	**H**	**14.59**	**9.50**
A	12.27	8.92	**F**	8.59	6.11	**G**	**17.34**	**10.70**
			K	10.80	8.04			
			E	8.52	5.50			
			R	9.97	7.90			

Table 9. Minimum inhibitory concentration (MIC) values of all nisin derivatives, N20P$^{1-28}$, M21V$^{1-28}$, K22T$^{1-28}$, N20P, M21V, K22T, nisin PV, N-PV, M-PV and T-PV against a range of NSR-expressing and non-expressing indicator organisms. Identical MICs values were obtained in three independent determinations. Bold font denotes enhanced activity compared to nisin PV.

	L. lactis MG1614	*L. lactis* MG1614 pNP40	*Streptococcus agalactiae* 67	*S. uberis* ATCC 700407
N20P $^{1-28}$ μg ml^{-1} (μM)	3 (0.937)	1.5 (0.468)	24 (7.5)	24 (7.5)
M21V $^{1-28}$ μg ml^{-1} (μM)	3 (0.937)	3 (0.937)	24 (7.5)	24 (7.5)
K22T $^{1-28}$ μg ml^{-1} (μM)	6 (1.875)	3 (0.937)	24 (7.5)	24 (7.5)
N20P μg ml^{-1} (μM)	0.125 (0.039)	0.5 (0.156)	3 (0.937)	6 (1.875)
M21V μg ml^{-1} (μM)	**0.065 (0.019)**	0.25 (0.078)	6 (1.875)	**1.5 (0.468)**
K22T μg ml^{-1} (μM)	0.125 (0.039)	0.25 (0.078)	24 (7.5)	3 (0.937)
PV μg ml^{-1} (μM)	0.125 (0.039)	0.125 (0.039)	3 (0.9375)	2 (0.625)
NPV μg ml^{-1} (μM	0.125 (0.039)	**0.0612 (0.019)**	**1.5 (0.468)**	3 (0.937)
MPV μg ml^{-1} (μM	0.25 (0.078)	0.125 (0.039)	12 (3.75)	**1.5 (0.468)**
TPV μg ml^{-1} (μM	0.5 (0.156)	0.125 (0.039)	12 (3.75)	3 (0.937)

Table 10. Area under the curve analysis of HPLC fractions corresponding to intact and truncated wild type nisin and nisin derivatives N20P, M21V and K22T.

	Intact	**Truncated**	**Total**	**%**
WT	69.98	22.64	92.62	24.44
N20P	107.02	11.80	118.82	9.93
M21V	62.41	14.30	76.71	18.64
K22T	82.96	16.77	99.73	16.81

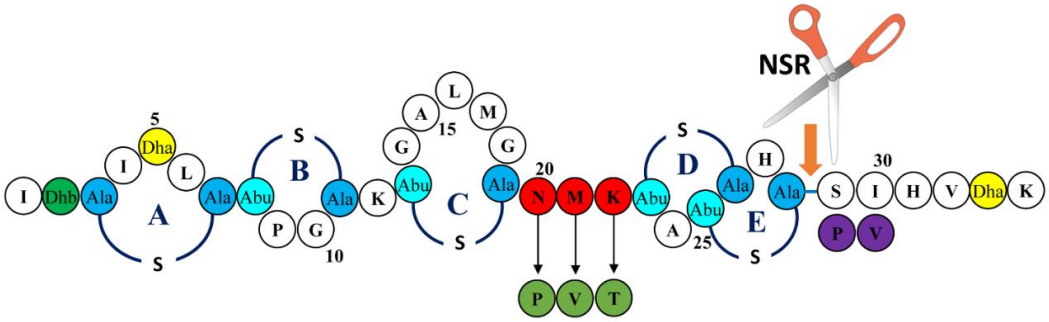


Figure 18. Structure of nisin A. Residues are represented in the single letter code. Post translational modifications are indicated as follows, Dha: dehydroalanine, Dhb: dehydrobutyrine, Abu: 2-aminobutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: 3-methyllanthionine. Green circles indicate amino acid differences between the natural nisin A and the hinge derivatives N20P, M21V and K22T. Purple circles denote the amino acid substitutions corresponding to the nisin PV derivative that is insensitive to the nisin resistance protein (NSR). The NSR cut-site is indicated by orange arrow.

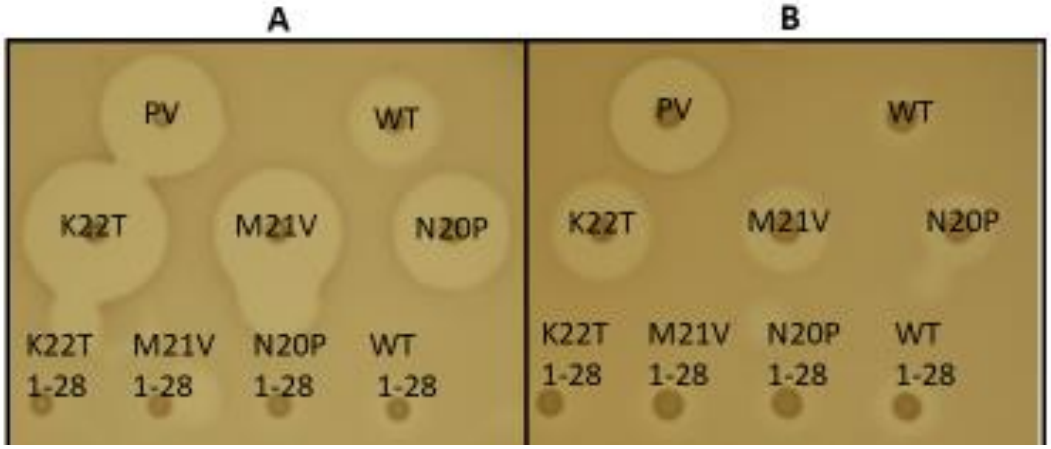


Figure 19. Deferred antagonism assay illustrating the zones of inhibition produced by strains of *L. lactis* NZ9800 producing nisin A (wt), the NSR insensitive derivative nisin PV, the hinge derivatives N20P, M21V and K22T and their truncated forms $N20P^{1–28}$, $M21V^{1–28}$ and $K22T^{1–28}$ against (A) *Lactococcus lactis* MG1614 (NSR-) and (B) *Lactococcus lactis* MG1614 (NSR+) which expresses the nisin resistance protein from the plasmid pNP40. All plates were supplemented with a sub-inhibitory concentration (100 ng/L) of nisin in the form of nisaplin to ensure induction of the truncated mutants and nisin PV.

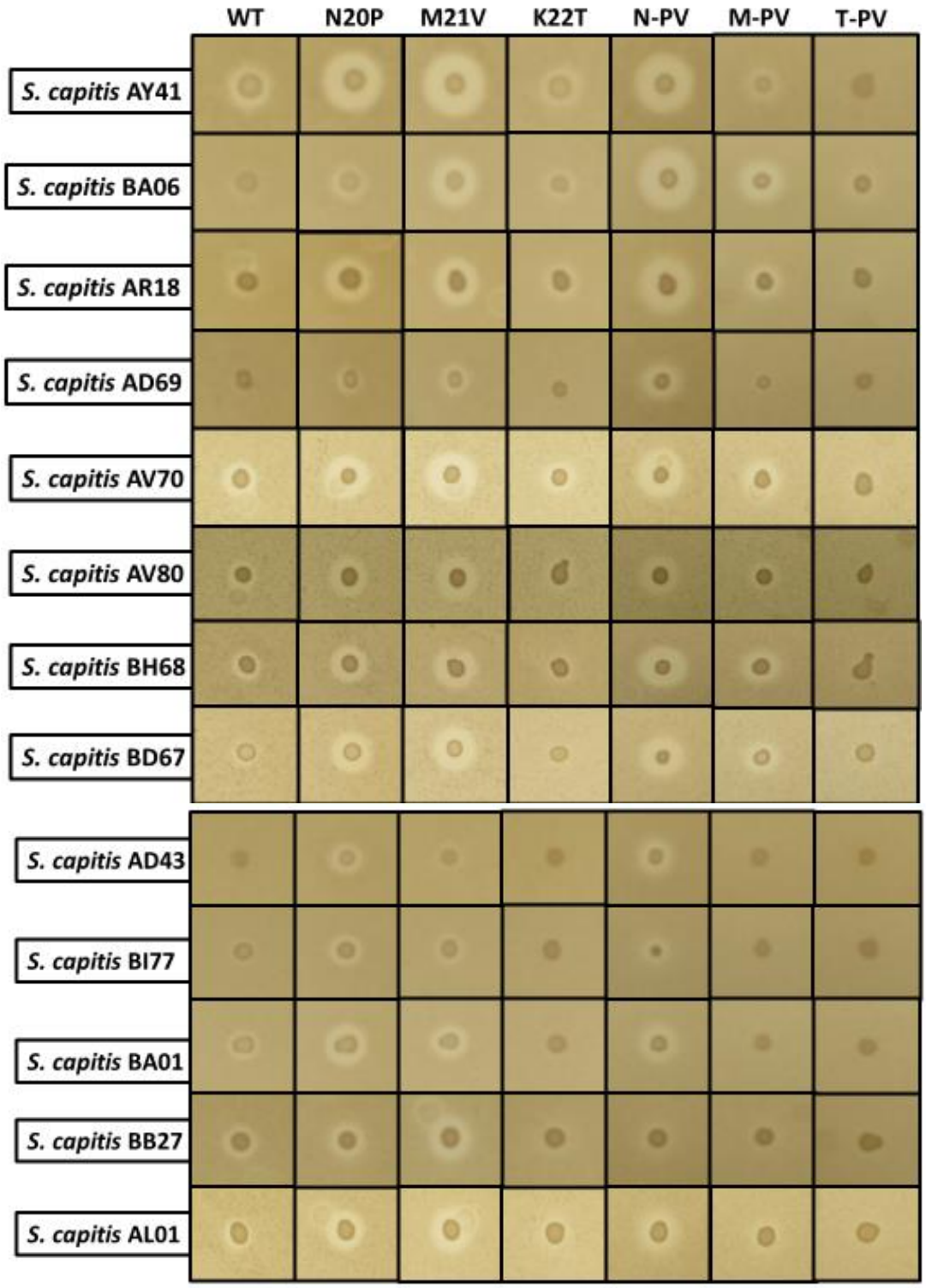


Figure 20. Deferred antagonism assay illustrating the zones of inhibition produced by the wildtype (WT) nisin A, hinge derivatives N20P, M21V and K22T and their combination variants N-PV, M-PV and T-PV against clinical *S. capitis* strains, all of which express the nisin resistance protein. All plates were supplemented with a sub-inhibitory

concentration (100 ng/L) of nisin in the form of nisaplin to ensure induction of the mutants.

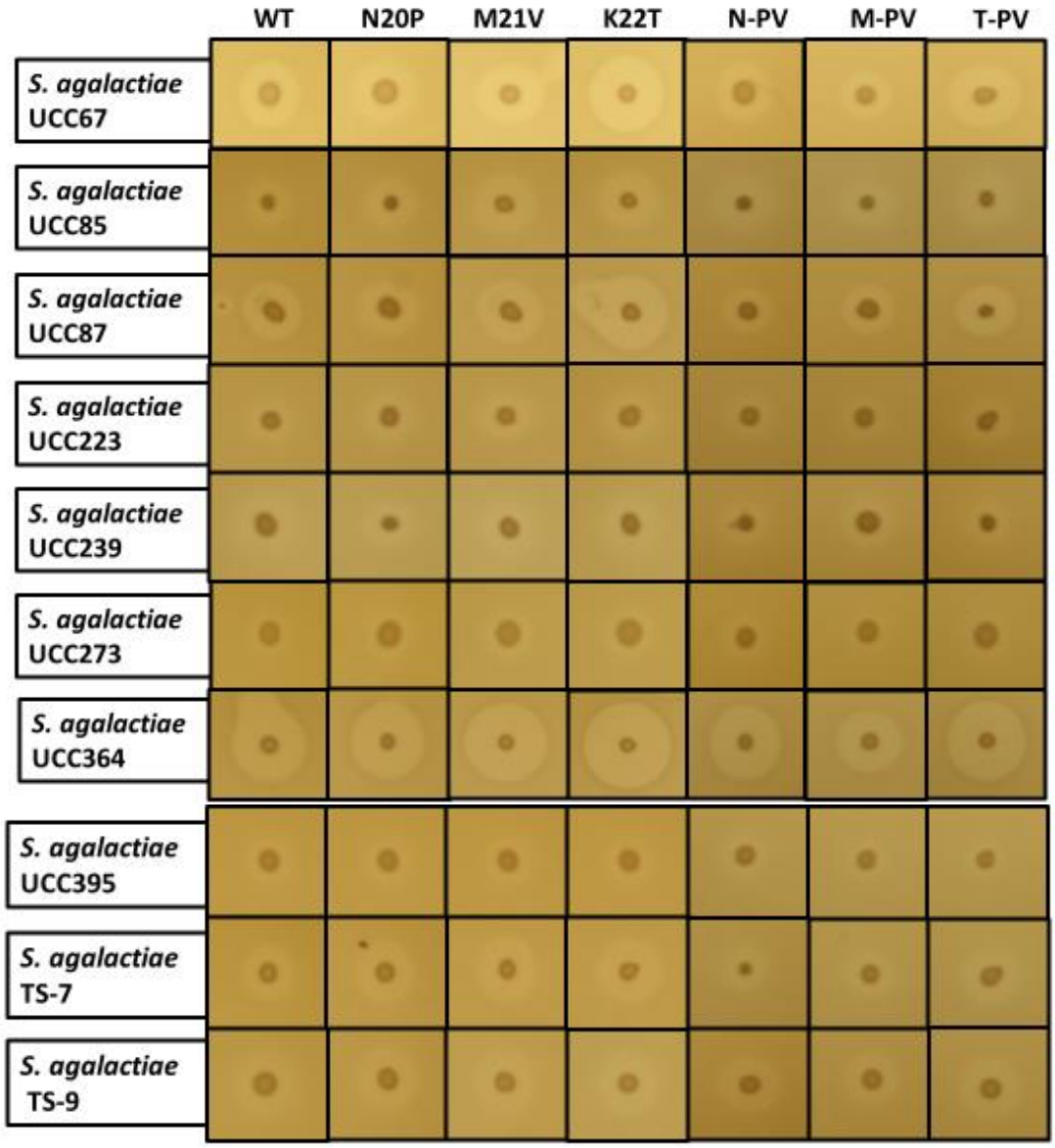
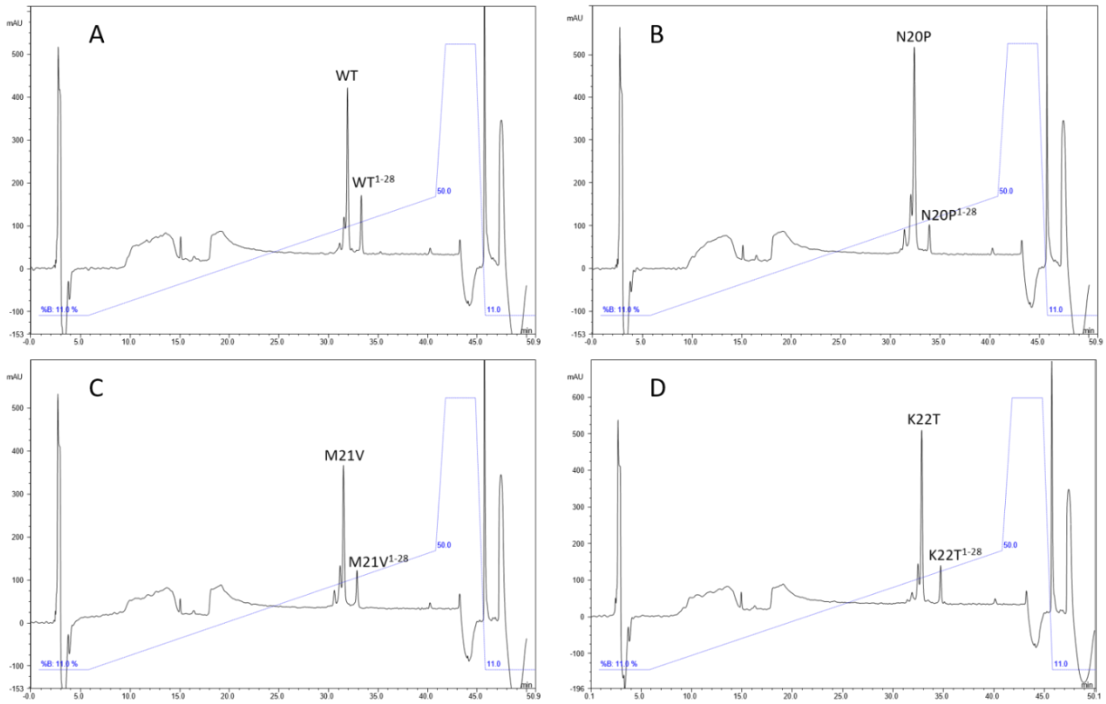


Figure 21. Deferred antagonism assay illustrating the zones of inhibition produced by the wildtype (WT) nisin A, the hinge derivatives N20P, M21V and K22T and their combination variants N-PV, M-PV and T-PV against clinical *S. agalactiae* strains, all of which express the nisin resistance protein. All plates were supplemented with a sub-inhibitory concentration (100 ng/L) of nisin in the form of nisaplin to ensure induction of the mutants.

1



2

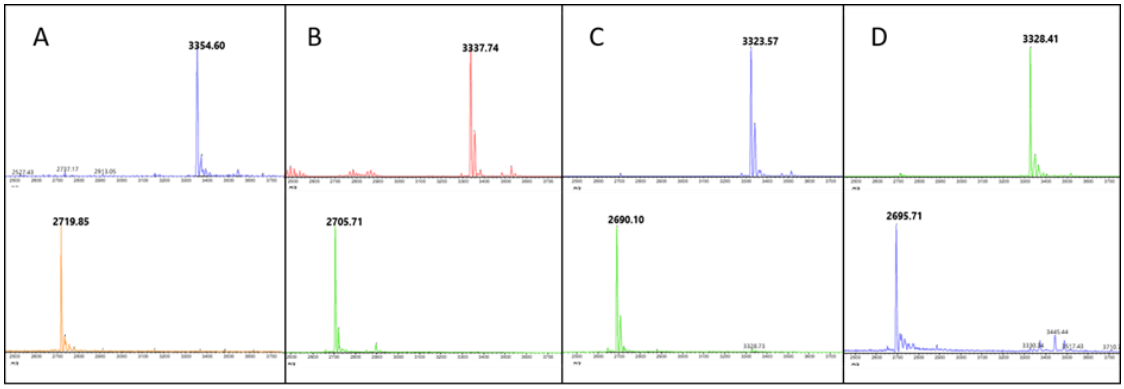


Figure 22. **(1)** Peptide release assay of nisin A and hinge derivatives N20P, M21V and K22T in the presence of NSR. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) analysis of (A) purified nisin A peptide following incubation with *L. lactis* subsp *diacetylactis* DRC3 (NSR+), (B) purified N20P peptide, (C) purified M21V peptide and (D) purified K22T peptide. **(2)** Mass Spectrometry analysis of HPLC fractions corresponding to major (top) and minor peaks (bottom) for (A) nisin A, (B) N20P, (C) M21V and (D) K22T.

Chapter 5

Thesis Conclusion

Thesis Conclusion

Bacteriocin research has expanded greatly over the past decade, with an explosion in the number of newly discovered novel bacteriocins. Indeed, it's hoped that bacteriocins will be developed to control food spoilage/pathogenic microorganisms, and to reduce multidrug-resistant clinical pathogens. Chapter 1 is a comprehensive review of current literature in relation to Staphylococcal-derived antimicrobial peptides. This review highlights the heterogeneous group of molecules with regard to size, charge, hydrophobicity, bioactivity and mode of action. Such is the heterogeneity, that there are considerable potential applications within the food, medical and veterinary industry to utilise these molecules to specifically target and eliminate a wide array of bacteria

To date, bacteriocins have been discovered from every conceivable niche, and it has been suggested that most bacteria produce at least one bacteriocin (Riley and Wertz, 2002). From this study (Chapter 2), we determined that antimicrobial production was prevalent among the CoNS strains with 94 out of 100 inhibiting the bacterial indicators, and 15 out of 94 strains inhibiting 10 or more indicators. This high prevalence indicates that antimicrobial production is a very useful trait for bacteria to have and will increase their fitness in order to be more competitive. Moreover, the high prevalence of antimicrobial production could suggest that horizontal gene transfer plays a significant role in dissemination of antimicrobial genes. Furthermore, most bacteriocins are encoded on plasmids that can be easily exchanged (Janek et al., 2016). This chapter also details the identification and characterization of a novel Class II bacteriocin from *S. capitis*, named capidermicin.

Following discovery and identification, the emphasis in this study (Chapter 3) turns to investigating whether capidermicin could inhibit *L. monocytogenes* under standard lab conditions. Firstly, capidermicin is a novel broad-spectrum class II bacteriocin, with remarkable stable at high temperatures and under acidic conditions. As a result of these attributes, we determined capidermicin has the potential to be used in conjunction with other bacteriocins, such as nisin, to prevent the growth of *L. monocytogenes* from foods. Here, we demonstrate the ability to provide even greater inhibition against *L. monocytogenes* by combining a class II leaderless bacteriocin with the class I lantibiotic nisin. Moreover, this interaction was shown to be maintained in a food matrix is noteworthy and highlights the potential of bacteriocin combinations to effectively control *L. monocytogenes* in food. The success of this peptide partnership can perhaps be attributed to their different modes of actions.

The development of nisin resistance among pathogens is an obstacle in the development of new food or therapeutic applications. The work described in this study (Chapter 4) confirms that lantibiotic peptides are quite adaptable and that bioengineering based approaches can be potentially rewarding with the generation of structurally novel antimicrobial nisin peptides. This investigation not only led to a better understanding of the nisin peptide and nisin resistance but resulted in the discovery and characterisation of the first enhanced combination nisin variant, as well as a nisin variant with potential applications in the treatment of problematic NSR clinical pathogens. Thus, the results presented in this thesis emphasize the benefits of bioengineering when performing fundamental analyses of lantibiotics. The continuation of rational mutagenesis strategies will be central to efforts to unlock the structure-function relationship within lantibiotics,

and thus provide the framework for the design and optimisation of more potent antimicrobial peptides to combat bacterial resistance.

To achieve the full potential of any bacteriocin in practical applications, it is important to have a better understanding of their activity, spectrum of inhibition, production and mode of action. Future work with capidermicin should focus on determining the mode of action and carry out a comprehensive analysis of the capidermicin gene cluster. Additional studies will be required to determine the potential application of capidermicin for use in food. Paramount will be the apparent synergistic relationship with nisin as a food preservative. The use of capidermicin in combination with bioengineered nisin derivatives which have been shown to possess enhanced activity against *L. monocytogenes* compared to nisin A could further enhance the safety of foods.

Indeed, the work described herein provides a significant amount of information with respect to bacteriocin discovery, most notably the high prevalence of antimicrobial compound production among CoNS strains, first report of a class II leaderless bacteriocin from *S. capitis* and its synergistic interaction with nisin to significantly reduced *L. monocytogenes* in food models.

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Appendices