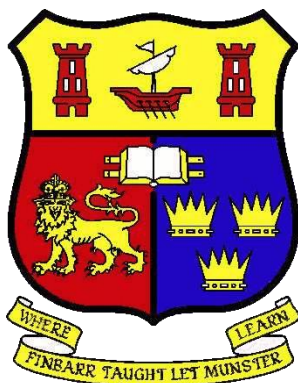


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Bioengineering of Nisin to Enhance Functionality against Dairy Pathogens



**A Thesis Presented to the National University of Ireland
for the Degree of
Doctor of Philosophy
By**

Brian Cornelius Healy, B.Sc.

**Student ID No. 109220278
December 2014**

School of Microbiology
University College Cork

Supervisors: Prof. Colin Hill, Dr. Paul Cotter and Prof. Paul Ross

Head of School: Professor Gerald Fitzgerald

For Mark, Helen and Grandad

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Declaration

I hereby declare that this thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed _____

Brian Healy

Publications

Healy B, O'Mahony J, Hill C, Cotter PD & Ross RP (2010) 2 Lantibiotic-related Research and the Application Thereof. *Antimicrobial Peptides: Discovery, Design and Novel Therapeutic Strategies* **18**: 22.

Healy B, Field D, O'Connor PM, Hill C, Cotter PD & Ross RP (2013) Intensive mutagenesis of the nisin hinge leads to the rational design of enhanced derivatives. *PLoS One* **8**: e79563

Abstract

It is a cause for significant concern that, as the threat of multi-drug resistant strains of bacteria has increased, the discovery of effective and novel antibiotics has stalled. The bacteriocin class of antimicrobial peptides have, in recent times, emerged as a viable alternative to at least partially fill the void created by the end of golden age of antibiotic discovery. Along with this potential use in a clinical setting, a number of bacteriocins also play an important role as bio-preservatives in the food industry.

This thesis focuses on a specific group of the bacteriocins, the Class 1 lantibiotics (**Lanthionine-containing antibiotics**). The mature peptide structure contains a number of unique amino acids which result from enzymatically mediated post-translational modifications, including dehydration and cyclisation, leading to the formation of the eponymous (methyl)lanthionine bridges. Two characteristics associated with lantibiotics are exploited in this work. Firstly, the many ways in which they can be applied in a food setting, and secondly, how their gene-encoded nature can be modified to improve on overall bioactivity and functionality.

The research focuses on two lantibiotics, lacticin 3147 and nisin A, both of which are produced by strains of *Lactococcus lactis*. The use of a lacticin 3147 producing starter culture with a view to the control of the Crohn's disease-linked pathogen *Mycobacterium avium* subspecies *paratuberculosis* was assessed in a smear ripened raw milk cheese. The fact that bacteriocin production did not effectively control the pathogen was one of a number of factors that provided an impetus to explore a variety of PCR-based mutagenesis techniques with a view to the

creation of enhanced lantibiotic derivatives. Initially there was a specific focus on the three amino acid central 'hinge' region of nisin A, chosen because previous bioengineering of this region led to variants with enhanced properties.

Through the use of both site specific and site directed mutagenesis techniques, a number of enhanced derivatives were generated. A derivative in which the three hinge amino acids (asparagine, methionine and lysine) were replaced with three alanines represents the first enhanced derivative of nisin to have been designed through a rational process. This derivative, named nisin AAA, also formed the backbone for the creation of an active, trypsin resistant, nisin variant. This stemmed from the observation that the lysine at position 12 provided the only trypsin sensitive peptide bond in nisin AAA. Introduction of a methionine residue at this position led to the creation of nisin K12M-AAA, which maintained antimicrobial activity while demonstrating resistance to the protease. This functional change could see nisin used as an oral anti-bacterial in the future as this derivative is more likely to be capable of withstanding gastric transit. A number of lead nisin derivatives were investigated to assess their anti- *Streptococcus agalactiae* ability. The ability of the AAA producer to control this target, which can contribute to the commercially important disease, bovine mastitis, was evident from these studies. Finally, a system was also developed to facilitate the large scale production of these candidates, or other nisin derivatives, from dairy substrates with a view to anti-mastitis or other therapeutic preparations.

Chapter I

Literature Review

Lantibiotic-related research and the application thereof

Brian Healy, Jim O'Mahony, Colin Hill, Paul D. Cotter and R. Paul Ross.

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1.1 Abstract

Since the discovery, in 1925, that strains of *E. coli* can retard the growth of neighbouring bacteria, the study of bacteriocins has evolved continuously. During the intervening period a large and heterogeneous, collection of these antimicrobial peptides has been isolated from a myriad of sources and numerous investigations have been carried with a view to harnessing this potency. The most thoroughly investigated class of bacteriocins, the lantibiotics, will be the main focus of this review. These antimicrobial peptides inhibit many human and animal pathogens and have been the focus of considerable efforts to maximise the potential of existing lantibiotics, identify new and better lantibiotics from nature and to utilise bioengineering-based approaches to further improve upon existing well characterised compounds.

1.2 Introduction to Bacteriocins

In order for an organism to survive, thrive and proliferate in a particular niche, it is essential that it competes successfully. In the microbial world many bacteria gain an upper hand by producing antimicrobial compounds which inhibit their competitors. Bacteriocins, which are bacterially-produced ribosomally-synthesized peptides with antimicrobial spectra which can range from very narrow to extremely broad, can be among the most potent examples of bacterially produced antimicrobials (Tagg *et al.*, 1976, Jack *et al.*, 1995, Riley & Wertz, 2002, Cotter *et al.*, 2005). Indeed, bacteriocins frequently exhibit antimicrobial activity at nanomolar levels which contrasts with, for example, the cationic antimicrobial peptides (CAMPs) produced by eukaryotic cells which only exhibit similar levels of activity at micromolar concentrations (Nissen-Meyer *et al.*, 2009).

The identification of the first bacteriocin, a colicin (i.e. an *Escherichia coli*-associated bacteriocin (Gratia, 1925)), occurred in 1925. This was followed soon after by the first report of a bacteriocin produced by a Gram-positive bacterium when inhibition of a *Lactobacillus bulgaricus* by a *Streptococcus lactis* (since renamed *Lactococcus lactis*) was noted (Rogers, 1928). Since then, bacteriocins produced by lactococci and other lactic acid bacteria (LAB), i.e. microorganisms which have a long history of safe use in food and are of considerable industrial importance, have continued to be the focus of much attention (Nissen-Meyer *et al.*, 2009). There are a number of different classification systems for LAB bacteriocins but the simplest, and that used for this review, was proposed by Cotter *et al.*, (Cotter *et al.*, 2005) and comprises two classes i.e. Class I bacteriocins: the post-translationally modified lantibiotics (Chatterjee *et al.*, 2005, Cotter *et al.*, 2005), and Class II bacteriocins, which are a heterogeneous group of non lanthionine-containing peptides. As a

consequence of their unusual structure, unique mechanisms of action and their potential as antimicrobials for food, veterinary and clinical application, the lantibiotics have been the target of much attention in recent years and are the focus of this chapter.

1.3 Biosynthesis and Post-Translational Modifications

The name lantibiotics is derived from *lan*thionine-containing *anti*biotics and reflects the feature which is shared by all of these peptides i.e. intra-molecular rings formed by the thioether amino acids lanthionine (Lan) and methyllanthionine (meLan) (Sahl *et al.*, 1995). Lantibiotic-associated gene clusters can be located on chromosomes (including transposable elements) or plasmids and in addition to the genes encoding a structural peptide(s) and the post-translational modification machinery that acts thereon, other genes responsible for regulation, transport and immunity are usually also present (Cotter *et al.*, 2005). Here the steps involved in lantibiotic biosynthesis are summarized.

The lantibiotic prepropeptide contains a leader region which is eventually cleaved generating propeptide which is modified to become the active antimicrobial. The corresponding gene is generically designated *lanA*. Lantibiotics can be further subdivided according to the enzymes which catalyse (me)Lan formation. Type I prepropeptides such as the prototypic lantibiotic, nisin A, undergoes dehydration as a result of the catalytic activity of the NisB protein (generically referred to as LanB enzymes) (Karakas Sen *et al.*, 1999). As a result specific serine and threonine residues are dehydrated to become the unique amino acids 2,3-dehydroalanine (Dha) and 2,3-dehydrobutyrine (Dhb), respectively. Some or all of these new residues are

then the subject of a LanC catalysed reaction which results in their interaction with the thiol groups of cysteines within the peptide to form Lan and meLan, from Dha and Dhb, respectively. Insight into the role of LanC was first provided by Meyer and co-workers (Meyer *et al.*, 1995) who, while working on the lantibiotic Pep5, showed that with the removal of the *pepC* gene, the peptide produced contained dehydrated residues but not lanthionine or methyllanthionine bridges. In addition to being essential for converting the peptide to an active form, the (me)Lan structures are also believed to contribute to protease resistance (Kluskens *et al.*, 2005). Unlike the Type I peptides which are modified by two separate modification enzymes, Type II peptides, such as lactacin 481 and mersacidin, are modified by a single protein referred to as LanM (Willey and van der Donk, 2007). Another variation on this theme arises as a consequence of the existence of two peptide lantibiotics, such as lactacin 3147, which require the combined activity of two (me)Lan-containing peptides for optimal activity. In the majority of cases the production of lantibiotics of this kind relies on the presence of two LanM proteins, each of which are responsible for the dehydration and cyclisation of their respective subunit (McAuliffe *et al.*, 2000, Lawton *et al.*, 2007). A third category of peptides, Type III, is not described in this review as those identified to date, while possessing (me)Lan structures, lack antimicrobial activity (Willey & van der Donk, 2007) while yet another category, modified by a novel group of lantibiotic synthetases designated LanL, has recently been discovered (Goto *et al.*, 2010). It remains to be established if LanL modified peptides possess antimicrobial activity and thus it has been suggested that the term lantipeptides be used to describe lanthionine-containing peptides that lack antimicrobial activity (Goto *et al.*, 2010). With respect to the lantibiotics, in addition to the LanB, C and M enzymes, a number of other modification enzymes have been

associated with the modification of specific peptides (Kupke & Gotz, 1997, Peschel *et al.*, 1997, Majer *et al.*, 2002, Cotter *et al.*, 2005).

After modification, the peptide is transported *via* an ABC transport system to the cell surface (Fath & Kolter, 1993) and the leader sequence is proteolytically removed, although not necessarily in that order, leading to the production of the active lantibiotic (Havarstein *et al.*, 1995). For Type I lantibiotics these individual steps are carried out independently by a transporter (designated LanT) (Qiao and Saris, 1996) and a protease (LanP)(Ye *et al.*, 1995, Siezen, 1996), whereas in the case of Type II lantibiotics both activities are carried out by one enzyme (also designated LanT) (Rince *et al.*, 1994, Chen *et al.*, 1999, Altena *et al.*, 2000). To ensure that the active antimicrobial does not target the producing cells, immunity proteins are produced. Two immunity mechanisms have been described. One relies on a single immunity protein, LanI, while a second involves a multi-component ABC-transporter system referred to as LanEFG. Indeed some lantibiotic producers utilize two such mechanisms (Draper *et al.*, 2008). The remaining group of lantibiotic-associated proteins to which we have yet to refer are the regulators. Initial studies in this area focused on the regulation of nisin, and its related peptide, subtilin by two component signal transduction systems which consist of a histidine kinase (LanK) and a response regulator (LanR) (Klein *et al.*, 1993, Engelke *et al.*, 1994, Stock *et al.*, 2000, Kleerebezem, 2004). Examination of the genetic determinants of a subtilin producer revealed the presence of the *spaK* and *spaR* genes. Using a strategy of gene deletion, it was shown that mutants lacking these genes were no longer capable of lantibiotic production (Klein *et al.*, 1993). A similar approach also revealed the importance of the corresponding nisin genes (Engelke *et al.*, 1994). In both cases, it has been established that the associated antimicrobial peptides also

function as pheromones as part of a LanRK-mediated quorum sensing system which facilitates autoregulation (Kleerebezem, 2004). While two component systems play a major role in the production of a number of other lantibiotics, such as mersacidin (Guder *et al.*, 2002), other quite different regulators have also been noted (McAuliffe *et al.*, 2001).

1.4 Classification

A scheme to classify lantibiotics was first proposed by Jung in 1991 (Jung, 1991). This scheme grouped the peptides into two distinct categories. The first class, Type-A, contained the elongated flexible, amphipathic, peptides. These peptides also had an overall positive net charge and were thought to function through the permeabilization of the cell membrane. The second class, Type-B, were grouped on the basis of being globular proteins, with a net negative or neutral charge and were thought to function by inhibiting sensitive cells by forming complexes with specific membrane components. While some of this information continues to be pertinent, investigations which have established that some lantibiotics act in multiple different ways, coupled with the identification of new and more diverse lantibiotics, makes the use of this system more difficult. This issue has been addressed by approaches that reflect the genes and proteins involved in lantibiotic production. Most recently this has involved the fusion of two compatible approaches (Piper *et al.*, 2009a) in which peptides were classified on the basis of the sequence of the lantibiotic propeptide (Cotter *et al.*, 2005) or the composition of the associated modification/ transport systems (Pag & Sahl, 2002, Willey & van der Donk, 2007). Using this combined approach the lantibiotics can be subdivided into the three types, i.e. I, II and III,

referred to above, and 12 subgroups. The subclassification of the type I and II lantibiotics into 12 subgroups is based on the alignment of the unmodified amino acid sequence of the structural peptide. In each case homology results in the classification of the lantibiotic in question together with the eponymous member of the group i.e. planosporicin, nisin, epidermin, streptin, Pep5, lactacin 481, mersacidin, ltnA2, cytolysin, lactosin S, cinnamycin and sublancin (Fig. 1.1). Thus, for example, the mersacidin subgroup includes RumB, plantaricin C, mersacidin, michiganin, actagardine, C55a, LtnA1, SmbB, bhtA-alpha, Plwa, PmnA1 and BhaA1, all of which are modified by LanM enzymes (i.e. are of Type II) and contain a number of residues that are conserved across the group. It should be noted that problems can arise when trying to add newly purified lantibiotics for which a structure, but not gene sequences, are available to this system. Thus, while it is apparent from its unusual structure and modifications that microbisporicin represents a 13th lantibiotic subclass (Castiglione *et al.*, 2008), it will not be possible to satisfactorily incorporate it into the system until the associated biosynthetic genes are identified and the *lanA* gene sequenced.

1.5 Lantibiotic subgroups – Biology, Structure and Mode of Action

1.5.1 Nisin-like lantibiotics

Nisin A is the most thoroughly characterized lantibiotic. This 34 amino acid peptide contains three dehydrated amino acids and five thioether rings (Fig 1.2) (Gross & Morell, 1971, Buchman *et al.*, 1988) and is the eponymous member of the nisin-like lantibiotics, which also include nisin Z (Mulders *et al.*, 1991), nisin F (de

Kwaadsteniet, *et al.*, 2008), nisin U and U2 (Wirawan *et al.*, 2006), nisin Q (Zendo *et al.*, 2003), subtilin (Klein *et al.*, 1993), ericin S and ericin A (Stein *et al.*, 2002). The mechanism of action of nisin A (and nisin Z, which differs from nisin A by one amino acid) has been investigated in depth and it is likely that all related peptides function in a broadly similar manner. In addition to a long standing appreciation of its ability to form pores, it was revealed in the mid-90s that nisin binds to lipid II, an essential precursor of peptidoglycan and thus also inhibits cell wall synthesis (Brotz *et al.*, 1998, Breukink *et al.*, 1999). Structural analysis of the lipid II-nisin complex has revealed that the N-terminal region of the nisin peptide is responsible for lipid II binding (Hsu *et al.*, 2004). The C-terminal end of the peptide, which is linked to the N-terminus by a three amino acid 'hinge' region, is the pore-forming domain (Breukink *et al.*, 1997, Wiedemann *et al.*, 2001). The efficiency of pore formation is greatly enhanced by lipid II-binding (Breukink *et al.*, 1999). A third mode of action has also been revealed in that it is now apparent that nisin can induce the autolysis of susceptible staphylococcal strains as a consequence of the release of two cell wall hydrolyzing cationic enzymes during normal autolysis of dividing cells (Hasper *et al.*, 2004).

1.5.2 Epidermin-like lantibiotics

Epidermin is produced by *Staphylococcus epidermidis* and is encoded by a structural gene, *epiA*, which is located on a 54 kb plasmid (Schnell *et al.*, 1992). The active lantibiotic is a 21 amino acid peptide which contains four rings including three (me)Lans and a 2-aminovinyl-D-cysteine (Allgaier *et al.*, 1986). After epidermin, gallidermin is the most extensively studied epidermin-like lantibiotic. The two peptides are structural analogues which differ by only one residue (at position

6)(Kellner *et al.*, 1988). NMR studies into the structure of gallidermin has revealed that the rigid N-terminally located rings A and B are connected to the C and D rings at the C-terminal end of the peptide by a somewhat flexible region spanning residues A12 to G15 (Ottenwalder *et al.*, 1995). A comparison of the structures of epidermin and nisin show a high homology in the N-terminal, lipid II-binding, ends of the peptides (Hsu *et al.*, 2004) whereas their C-terminal ends differ considerably, thereby explaining the inability of epidermin to form pores in certain targets (Bonelli *et al.*, 2006).

1.5.3 Planosporicin- and Streptin-like lantibiotics

In addition, to the nisin- and epidermin-like peptides, there exist two other peptides, planosporicin and streptin, which resemble the former with respect to their N-terminal domains but differ quite considerably from these, and from one another, otherwise. As a result both are the eponymous (and sole) members of two lantibiotic sub-classes. Planosporicin, produced by *Planomonospora* sp., contains both Lan and meLan residues which generate five intramolecular thioether bridges (Fig 1.2) (Castiglione *et al.*, 2007, Maffioli *et al.*, 2009). Planosporocin functions primarily through the inhibition of peptidoglycan synthesis but unlike nisin and epidermin does not bind to the D-ala-D-ala motif of lipid II (Castiglione *et al.*, 2007). Streptin is a *Streptococcus pyogenes*-associated lantibiotic, two major forms of which have been purified. Streptin 1 is the fully mature 23 amino acid peptide while a second form, streptin 2 has three additional residues at the N-terminal end (TPY)(Karaya *et al.*, 2001, Wescombe & Tagg, 2003).

1.5.4 Pep5-like peptides

Pep5 is a tricyclic lantibiotic consisting of 34 amino acids (including 3 lanthionine bridges; one meLan and two Lan)(Bierbaum *et al.*, 1994, Bierbaum *et al.*, 1996) and is both screw shaped and highly cationic, the latter being due to the presence of six lysine and two arginine residues in the mature peptide (Pag *et al.*, 1999). It is produced by *Staphylococcus epidermidis* strain 5 (Kaletta *et al.*, 1989) with the associated genes being located on the 20 kb plasmid pED503 (Ersfeld-Dressen *et al.*, 1984, Meyer *et al.*, 1995). Pep5 also has another less common feature i.e. an N-terminal 2-oxobutyryl group which is thought to be formed through the non-enzymatic hydrolysis of N-terminal Dhb residues (Xie & van der Donk, 2004). Pep 5 exerts its mode of action by forming voltage dependant pores in the cytoplasmic membrane of sensitive cells (Sahl *et al.*, 1987, Kordel *et al.*, 1989). These pores then cause the leakage of essential metabolites and ATP out of the cell resulting in the cessation of cellular metabolic processes and ultimately causing cell death. Pep5 can also induce the autolysis of staphylococci through the activation of cell wall hydrolysing enzymes (Bierbaum & Sahl, 1985, Bierbaum & Sahl, 1987).

1.5.5 Lacticin 481-like lantibiotics

The Type II lacticin 481-like subgroup is the largest lantibiotic subgroup and the associated peptides are also notable by virtue of lacking post-translational modifications other than the common Dha, Dhb, Lan and meLan residues (Dufour *et al.*, 2007). The *Lactococcus lactis* ssp. *lactis* CNRZ481-produced eponymous member of this group, lacticin 481, is 27 amino acids in length and contains two Lans, one meLan and one Dhb residue (Fig 1.2)(Piard *et al.*, 1993, van den Hooven *et al.*, 1996). Interestingly, although lacticin 481 is quite similar to the related variacin (five amino acid differences), variacin appears to have a much broader

target-cell spectrum of activity (Pridmore *et al.*, 1996). Studies with another member of this group, nukacin ISK-1, indicate it to be bacteriostatic and incapable of pore formation (Asaduzzaman *et al.*, 2009) whereas the related streptococcin SA-FF2 peptide causes the formation of short-lived pores in target cells (Jack *et al.*, 1994).

1.5.6 Mersacidin-like lantibiotics

Mersacidin is a small lantibiotic produced by *Bacillus* spp. (Chatterjee *et al.*, 1992). The biosynthetic gene cluster (12.3 kb in size) consists of 10 genes and is located on the bacterial chromosome. This hydrophobic and neutral peptide contains three meLans and a single 2-aminovinyl-2-methylcysteine corresponding to four intramolecular rings (Fig 1.2)(Chatterjee *et al.*, 1992). Mersacidin does not form pores in the cell membrane of sensitive cells but does inhibit cell wall synthesis through binding with lipid II (Brotz *et al.*, 1995). An NMR study carried out by Hsu and co-workers showed that mersacidin can change its conformation depending whether or not it is in the presence of lipid II (Hsu *et al.*, 2003). In addition to one peptide lantibiotics such as mersacidin, the mersacidin-like peptides also contain the A1 peptide of a number of two peptide lantibiotics i.e. lactacin 3147 (Fig 1.2)(Ryan *et al.*, 1999), staphylococcin C55 (Navaratna *et al.*, 1998), plantaricin W (Holo *et al.*, 2001), BHT (Hyink *et al.*, 2005), Smb (Yonezawa & Kuramitsu, 2005), licheniciden (Begley *et al.*, 2009, Dischinger *et al.*, 2009) and haloduricin (Fig 1.2)(Mc Clerren *et al.*, 2006, Lawton *et al.*, 2007). These two peptide lantibiotics only exhibit optimal activity when the A1 component is combined with its A2 counterpart. These conserved A2 peptides are referred to as the LtnA2-like peptides and are described below.

1.5.7 LtnA2-like peptides

Lacticin 3147 is the most extensively studied two peptide lantibiotic and is produced by *Lactococcus lactis* subsp. *lactis* DPC3147 (Ryan *et al.*, 1996). The genes responsible for the production of the lantibiotic, and of the associated immunity proteins, are encoded on a 60.2 kb conjugative plasmid, pMRC01, which contains 10 ORFs (Dougherty *et al.*, 1998). The mechanism of action of lacticin 3147, which is also lipid II mediated, depends on the presence of both components i.e. Ltn α and β (derived from the LtnA1 and LtnA2 propeptides, respectively). More specifically, Ltn α first binds to lipid II in sensitive cells. This binding is thought to lead to a conformational change which produces a high affinity binding site for the Ltn β peptide. Cell death occurs through the permeabilization of the cell membrane (Wiedemann *et al.*, 2006) leading to the efflux of K⁺ ions and phosphate resulting in hydrolysis of cellular ATP. The related haloduracin peptide has recently been shown to function in a similar way (Oman and van der Donk, 2009)

1.5.8 Other Type II Lantibiotics

Other type II subgroups include the cinnamycin-like, sublancin-like, lactocin S-like and cytolysin-like subgroups. While there are a number of cinnamycin-like peptides, sublancin, lactocin S and cytolysin are the sole members of their respective subgroups. Cinnamycin is a tetracyclic lantibiotic produced by *Streptoverticillium grisoverticillutum*. It is a 19 amino acid peptide and contains the unusual residues lysinoalanine and 3-hydroxyaspartic acid (Fig 1.2). In addition to its antimicrobial activity, cinnamycin and related peptides have other potentially useful

pharmaceutical properties, including the inhibition of phospholipase A2 and angiotensin converting enzyme (Fredenhagen *et al.*, 1990, Kaletta *et al.*, 1991, Hosoda *et al.*, 1996). Sublancin, produced from *B. subtilis* 168, is a 37 amino acid peptide which contains one meLan and two disulphide bridges. The presence of the stabilizing lanthionine bridges along with relatively weak disulphide bridges suggests a conformational uniqueness which could confer a selective advantage (Paik *et al.*, 1998). Lactocin S, produced from *L. sake* L45, is a 37 amino acid lantibiotic (Mortvedt *et al.*, 1991, Skaugen *et al.*, 1997). This lantibiotic is noteworthy as a consequence of containing D-alanine residues (Skaugen *et al.*, 1994). Among lantibiotics only lactacin 3147 shares this trait. At a neutral pH, this lantibiotic exhibits a net neutral charge (Rawlinson *et al.*, 2002). Finally, cytolysin is a two peptide lantibiotic (Cyl_{LL} and Cyl_{LS}) produced by enterococci which is unique by virtue of its ability to target both eukaryotic and prokaryotic cells (Booth *et al.*, 1996).

1.6 Current and Future Use of Lantibiotics for Food Applications

The most prominent event in the majority of food fermentations is the conversion of sugars to lactic acid by the LAB. In addition to lactic acid, LAB can produce other metabolites with antimicrobial activity such as hydrogen peroxide, diacetyl, acetoin and bacteriocins, including a number of lantibiotics such as nisin A. Highlights in the industrial application of nisin A as a food preservative include its initial use by the food industry in 1953 and its approval by the World Health Organization, European Union and US FDA in 1969, 1983 and 1988, respectively.

Currently, nisin A has been approved for use in over 48 countries worldwide (Delves-Broughton *et al.*, 1996, Cotter *et al.*, 2005, Deegan *et al.*, 2006). There are a number of ways via which nisin can be added to a food. This includes the direct application of the peptide, in a highly purified form if necessary, as an ‘additive’ (being one of only two authorised, natural food antimicrobials; the other being the anti-mould additive natamycin), the introduction of a nisin-producing bacteria, as a starter or an adjunct culture, to a fermented food (and the subsequent production of nisin *in situ*) or the producer can be used to make a food-grade fermentate, which can be dried to make a powdered ‘ingredient’. This powder can then be incorporated into either fermented or non-fermented foods. In the case of nisin, all three alternatives are employed e.g. ‘Additive’ Nisaplin (from Danisco; <http://www.daniscocare4u.com/nisaplin.html>), Nisin-producing cultures (from culture providers such as Christian Hansen and CSK) or ‘Ingredient’ DURAfresh® (from Kerry).

Other than nisin, lacticin 3147 and lacticin 481 are the two most extensively studied LAB lantibiotics. Both exhibit traits that suggest they have commercial value and a few selected examples of potential applications are presented here. In the case of lacticin 3147 it has been established that lactococcal producers thereof can be employed as starter cultures for the manufacture of cheddar cheese (Ryan *et al.*, 1996). Here lacticin 3147 producers successfully reduce the pH of the milk to 5.2 while also generating sufficient quantities of the lantibiotic to control the adventitious non-starter lactic acid bacteria (NSLAB) over a six-month ripening period (Ryan *et al.*, 1996). This is significant as NSLAB can be the cause of flavour defects and calcium lactate formation (Thomas & Crow, 1983). The use of lacticin 481-producing strains as adjunct cultures in cheese production has also been mooted

(O'Sullivan *et al.*, 2003). This lantibiotic, which is produced by *Lactococcus lactis* strains (Piard *et al.*, 1992), demonstrates a higher ability to lyse sensitive lactococci than lacticin 3147 when used in combination with the starter culture *L. lactis* HP. The associated benefits are the release of intracellular enzymes, thereby speeding up the ripening process, and a reduction in the numbers of NSLAB (O'Sullivan *et al.*, 2003). Studies investigating the use of a lacticin 3147-based powder as a bio-preservative have also yielded interesting results (Morgan *et al.*, 2001). Like nisin, a fermentate containing lacticin 3147 provides an alternative means of introducing the lantibiotic into food. Incorporation of a whey powder (10%), which was fermented with a lacticin 3147 producer, has been found to bring about a 99.9% reduction in *Listeria monocytogenes* in natural yoghurt and an 85% reduction in pathogen numbers in a cottage cheese sample within a two hour time frame. It has also been established that an 80% reduction in *Bacillus cereus* numbers occurs within three hours when this powder is added (1% powder) to soup. For a comprehensive review on the use of bacteriocins as biological agents for food safety see Deegan *et al.* (2006).

1.7 Lantibiotics and their medical applications

The possibility of using lantibiotics to control or treat multi-drug resistant forms of pathogens such as *Staphylococcus aureus*, *Enterococcus* species and *Clostridium difficile* has gained increased attention in recent years due to a number of positive results obtained by researchers in the field (for a comprehensive review please refer to Piper *et al.* (2009a). *In vitro*, many lantibiotics, including lacticin 3147, mutacin B-Ny266, nisin, mutacin 1140, show activity against clinical targets

such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), *Propionibacterium acne*, *Streptococcus mutans*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Clostridium difficile*, *Listeria* and *Bacillus* species (Severina *et al.*, 1998, Galvin *et al.*, 1999, Mota-Meira *et al.*, 2000, Brumfitt *et al.*, 2002, Rea *et al.*, 2007, Ghobrial *et al.*, 2009, Piper *et al.*, 2009b). It is also interesting to note that both Pep 5 and epidermin successfully inhibit the adhesion of staphylococcal cells to the surfaces of catheters (Fontana *et al.*, 2006). It is important to note, however, that these represent just a selection of the studies which have highlighted the efficacy of lantibiotics against Gram-positive clinical pathogens. It is anticipated that the number of studies in this area will continue to increase as a consequence of the further investigation of existing lantibiotic peptides and the continued identification of new forms of these antimicrobials. Some recent examples of note include the two peptide lantibiotic, lichenicidin, which exhibits antimicrobial activity against MRSA and VRE strains (Begley *et al.*, 2009) and microbisporicin, active against MRSA, VRE and clinical streptococci (Castiglione *et al.*, 2008).

While the *in vitro* success of a chemotherapeutic agent does not always correspond to its *in vivo* efficacy, there have been a number of studies which indicate that this may not be a major failing of lantibiotics. It has been revealed that mutacin B-Ny266 can be as active as vancomycin against MRSA *in vivo* (Mota-Meira *et al.*, 2005), mersacidin can be employed to eradicate a nasal MRSA infection (Kruszewska *et al.*, 2004) and that nisin F, both alone and when used in combination with lysozyme and lactoferrin, can successfully treat respiratory tract MRSA infections in mice (De Kwaadsteniet *et al.*, 2009). Trials investigating the use of lantibiotics to control the microorganisms responsible for dental plaque, halitosis,

‘strep’ throat (Hillman, 2002, Burton *et al.*, 2006, Dierksen *et al.*, 2007) and even bovine mastitis (Ryan *et al.*, 1999, Twomey *et al.*, 2000) have also all been successful.

1.8 Engineering of lantibiotics

Lantibiotics are gene encoded and thus one can take advantage of this trait in order to engineer novel variants of the parent peptide. Such variants have been used to study structure/ function relationships and, in some cases, engineering strategies have led to the generation of peptides with enhanced antimicrobial activity. Lantibiotic engineering can take place *in vivo*, i.e. by manipulating the original producing strain or expressing the genes heterologously in an alternative host, or *in vitro*, i.e. harnessing the activity of purified forms of the individual components of biosynthetic machinery outside of a host. The application of engineering in lantibiotic research commenced in 1992. Although initial nisin-focused investigations did not lead to the production of variants with enhanced activity (Kuipers *et al.*, 1992), they clearly demonstrated the power of this technology. During the same year the extreme consequences of making single deliberate amino acid changes was demonstrated when it was established that a single residue change in subtilin resulted in a 57-fold increase in its biological and chemical stability (Liu & Hansen, 1992). This technology has continued to be applied and in 2006 the first alanine-scanning mutagenesis of a lantibiotic, lacticin 3147, was completed (Cotter *et al.*, 2006). In this study, alanine (or glycine in cases where an alanine was already present) was introduced in place of the 59 amino acids, in turn, and the impact on the antimicrobial activity of the associated producing strain was quantified. The data generated highlighted specific areas of the peptides in which subsequent site specific

mutagenesis approaches might be beneficial. This strategy was taken a step further when site-saturation mutagenesis was employed to engineer both nukacin ISK-1 (Islam *et al.*, 2009) and mersacidin (Appleyard *et al.*, 2009). Both studies provided an in-depth insight into the structure-function relationships within the respective peptides and, in the case of nukacin ISK-1, two variants which displayed a two-fold increase in specific activity were identified (Islam *et al.*, 2009). The use of engineering to study/improve nisin has also continued at pace and since the 1990s this lantibiotic has been the subject of a number of engineering-based strategies which have employed site-directed, site-saturation and/ or random mutagenesis. While various different regions of the peptide have been engineered, the N-terminal and 'hinge' regions have been the focus of greatest attention. The benefits of manipulating the hinge (consisting of Asn20-Met21-Lys22) have been particularly notable (Yuan *et al.*, 2004, Field *et al.*, 2008). Yuan and co-workers employed a site-directed approach whereby either positively or negatively charged amino acids were introduced into the hinge. These studies demonstrated that specific changes (i.e. N20K and M21K) increased the activity of the peptide against Gram-negative bacteria such as *Shigella* species., *Pseudomonas* species. and *Salmonella* species. In the case of Field *et al.*, screening of a bank of random mutagenised nisin variants revealed that a K22T variant displayed enhanced activity against the mastitic pathogen *Streptococcus agalactiae*. This prompted the use of site-saturation mutagenesis (Field *et al.*, 2008) for each of the individual hinge residues which, when coupled with a larger selection of target strains, lead to the identification of a number of peptides with enhanced activity against *Streptococcus agalactiae*, *Staphylococcus aureus* and *Listeria monocytogenes*. Yet another study, focusing on rings A and B at the N-terminal end of nisin A, showed that the various activities of

nisin can be altered by changing the amino acid arrangement in this region of the peptide (Rink *et al.*, 2007). Two mutants, designated KFI and KSI (letters indicate the amino acids present at positions 4, 5 and 6), displayed increased antimicrobial activity against a number of bacteria such as *Leuconostoc mesenteroides*, *Lactobacillus johnsonii* and *Lactococcus lactis*. KFI, and another variant VFG, also inhibited the outgrowth of *Bacillus subtilis* 168 spores more effectively than the wild-type.

It should be noted that these and other (bio)engineering-related strategies have also been used for a variety of other purposes such as increasing lantibiotic production (Cotter *et al.*, 2006, Heinzmann *et al.*, 2006), introducing lanthionines into class II bacteriocins (Majchrzykiewicz *et al.*, 2010) and even the post-translational modification of other bioactive peptides (Kuipers *et al.*, 2004, Kluskens *et al.*, 2009, Rink *et al.*, In Press). In addition to these approaches, the ever-improving ability of chemists to generate lantibiotic-like peptides through synthetic chemistry (Cobb & Vederas, 2007, Arnusch *et al.*, 2008, Ross *et al.*, 2010) is particularly exciting and has already facilitated the creation of potent nisin-vancomycin hybrids (Arnusch *et al.*, 2008).

1.9 Screening for new lantibiotics

While scientists are continuing with their efforts to further improve known lantibiotics, there is still considerable merit attached to identifying new peptides. It has also become apparent in recent years that the use of bioinformatics can be a very useful means of screening for such novel lantibiotics. The availability of databases, including both general (NCBI) and dedicated (BAGEL and BACTIBASE (de Jong *et*

al., 2006, Hammami *et al.*, 2007, Hammami *et al.*, 2010)), has facilitated the use of *in silico* approaches to lantibiotic screening. The two-peptide lantibiotic lichenicidin (Begley *et al.*, 2009) was identified using such an approach. Here, the highly conserved nature of the *lanM* gene was exploited to screen the ever increasing number of bacterial genome sequences that are publicly available. Initial screening revealed 89 *lanM* genes of which 61 had not previously been associated with lantibiotic production. One of the potential novel lantibiotic producers identified, *B. licheniformis* ATCC 14580, was selected and from it lichenicidin was isolated (Begley *et al.*, 2009). A similar approach was previously employed to identify another two-peptide lantibiotic, haloduracin, which is produced by *Bacillus halodurans* C-125 (McClerren *et al.*, 2006, Lawton *et al.*, 2007). Bioinformatics has also been of considerable use when designing engineered lantibiotics and novel lanthionine-containing peptides. A study by Rink and co-workers (Rink *et al.*, 2005) used bioinformatics to predict the impact of flanking amino acids on the dehydration of serine and threonine residues, and subsequent (me)Lan formation, in lantibiotic peptides. An *in silico* comparison of known lantibiotics found that the majority of modified serines and threonines were flanked by hydrophobic residues. *In silico* models predicted the likely impact of specific residues on modification when located adjacent to hydroxyl-amino acid residues. The subsequent creation, and investigation, of these peptides validated this theory.

The discovery of the lantibiotics microbisporisin and planosporicin was achieved using a more traditional screening method (Castiglione *et al.*, 2007, Castiglione *et al.*, 2008) involving 120,000 broth extracts obtained by fermenting 40,000 actinomycetes. Firstly the microbial products were screened to assess their activity against *S. aureus* before then selecting those which retained activity

following exposure to a β -lactamase cocktail or D-alanyl-D-alanine affinity resin i.e. were neither β -lactams nor vancomycin-like glycopeptides. Those which retained antimicrobial activity after these steps were selected for further investigation. This strategy yielded thirty-five lantibiotics, of which five showed little or no similarity to any known lantibiotics.

1.10 Outlook

The lantibiotic class of bacteriocins has the potential to be employed in a wide variety of different ways. As outlined above they have shown great promise as chemotherapeutics that can target the multi-drug resistant Gram-positive clinical pathogens, including the particularly problematic pathogens MRSA, VRE and *Clostridium difficile*. A number of these peptides are particularly attractive as a consequence of research that has established that they have mechanisms of action and target binding sites that are distinct from those of non-lantibiotics. This coupled with high potency and their generally non-cytotoxic nature could lead to these compounds having clinical applications in the future. The possibility of engineering new and improved lantibiotics, producing novel chemotherapeutics through the fusion of lantibiotics with antibiotics, the introduction of the lantibiotic-associated modifications into non-lantibiotics and the chemical synthesis of new lantibiotic-like peptides, as well as the ongoing use of traditional and *in silico* strategies to find novel compounds, all bring this potential to a new level. In addition to these new markets, it should not be forgotten that a lantibiotic, nisin, has been successfully employed by the food industry for over a half century. Other lantibiotics have the potential to be similarly employed and, as a consequence of the limited activity of nisin against certain target strains and species, together with its poor activity at

neutral pH, there are obvious niche-markets that these can fill. The commercial potential of lantibiotics and lantibiotic-related technology and the cutting edge fundamental science that underpins lantibiotic research will ensure that these peptides will continue to attract great attention in the coming years.

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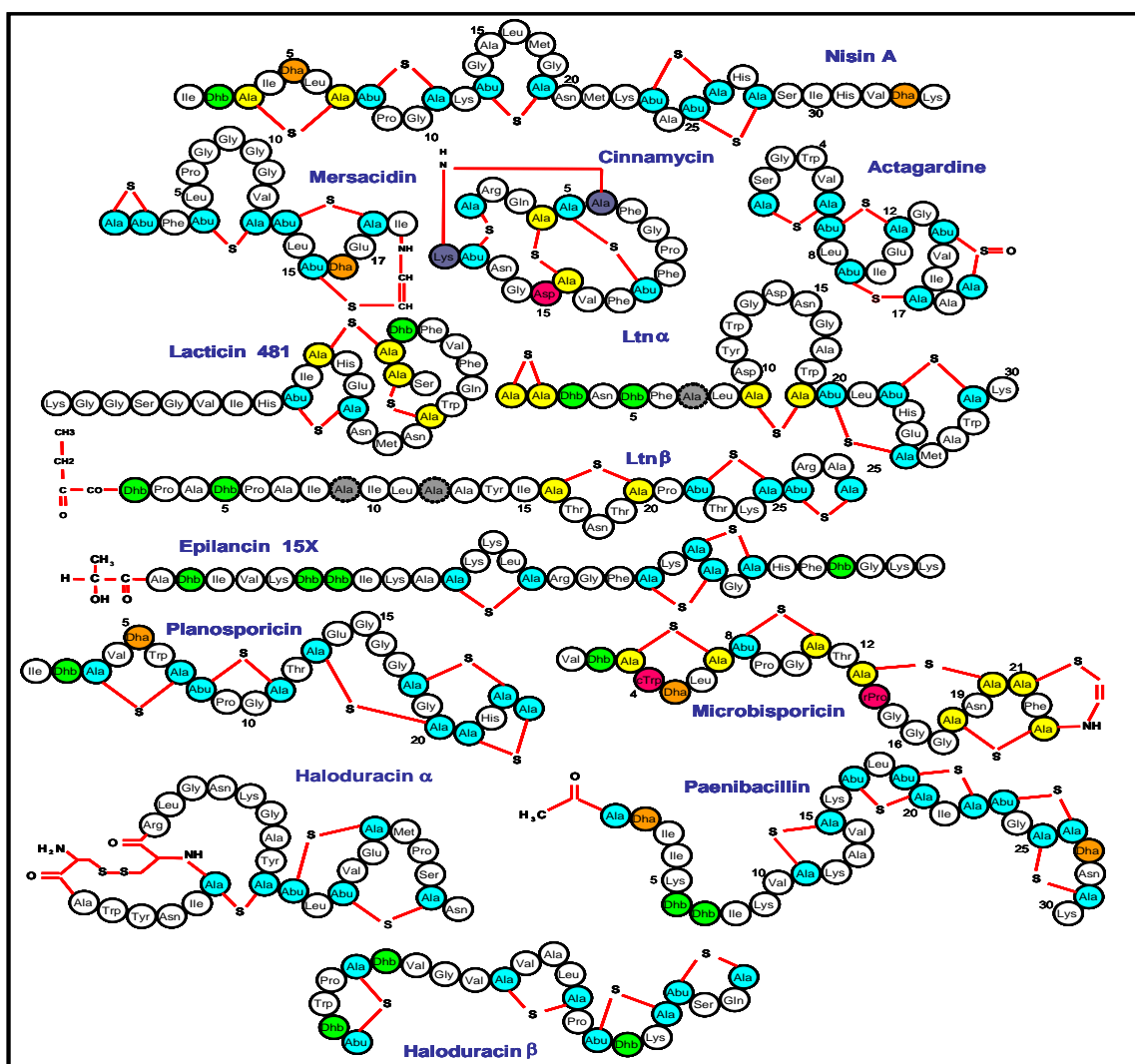
1.13 Figures

Figure 1.1 Lantibiotic classification

Class I (LanBC)	Planosporicin	Mersacidin	RumB	G E P R V S	D G S	Q F	C T S	T K E	N W G T I M F V C C
	Nisin		Plantaricin C		K K T K K N X S G D I		C T L	T S E	D H L A T W V C X
	Epidermin		Mersacidin	C T F T L P		G G G G V	C T L	T S E	I C
	Streptin		Michiganin			S G W L	C T L	T I Q	G I I A R
	Pep5		Actagardine		C	S G W V	C T L	T I E	G T V I C A C
	Mersacidin		LtnA1	C S T N T F S	L S D Y W G N N G A W		C T L	T H E	M A W C K
	LtnA1		SmbB	I G T T V V N S T F S I V L G N K G V I			C T V	T V E	M R N C S K
	Cytolysin		bhtA-alpha	I G T T V V N S T F S I V L G N K G V I			C T V	T V E	M R N C Q
	Lactocin S		Phwa	K C K W W N I S C D L G N N G H V			C T L	S H E	Q V S C N
Class III	Cinnamycin		BliA1	T I T L S T C A I L S K P L G N N G A V			C T V	T K E	M P S C N
	Sublancin		BhaA1	C A W Y N I S C R L G N K G A Y			C T L	T V E	M P S C N

Lantibiotics can be divided into Type I, II and III peptides. The Type I and II peptides can be further divided into 12 groups on the basis of the sequence of the lantibiotic propeptide. In one representative case, the mersacidin-like peptides, each of the corresponding propeptides are listed and aligned. Amino acids that are 100% conserved are highlighted in black.

Figure 1.2 Representative lantibiotic structures



Modified residues are coloured (dehydrobutyrine – Dhb, green; dehydroalanine – Dha, orange; lanthionineAla-S-Ala, yellow; β -methylanthionine – Abu-S-Ala, light blue; D-alanine – Ala, grey; lysinoalanine – Lys-NH-Ala; other modified residues - pink).

Chapter II

Fate of *Mycobacterium avium* subspecies *paratuberculosis* ATCC19698 during Manufacture and Ripening of a Non-Pasteurised Smear Type Cheese made with and without Lacticin 3147.

Brian Healy, Mary C. Rea, R. Paul Ross, Colin Hill and Paul D. Cotter

2.1 Abstract

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is an obligate pathogenic bacterium which is the causative agent for Johne's disease, a localized chronic infection of the lower intestine (ileum) of animals. *MAP* has also been implicated in Crohn's disease in humans, although this association remains highly controversial. There has also been considerable debate as to whether or not the microorganism can withstand HTST (High Temperature Short Time) pasteurization. Therefore, until it can be definitively established that the consumption of *MAP* infected food does not lead to human infections, the elimination of this pathogen from the food chain would be prudent.

In this study, an additional hurdle was introduced into a dairy product in order to determine if it provided additional protection against *MAP*. More specifically, a lacticin 3147 producing starter culture was employed for the production of smear ripened raw milk cheese. Lacticin 3147 is a two peptide bacteriocin (Bac) produced by a number of *Lactococcus lactis* strains, has been comprehensively characterised and has been shown to inhibit many clinically relevant Gram-positive pathogens, including *MAP*. Two separate cheeses were produced using either Bac⁺ or Bac⁻ versions of the same starter culture. Both were allowed to ripen for 4 weeks after the addition of the smear and along with the 'gold standard' plate enumeration method, the viability of the pathogen was also determined using a molecular approach.

Although some decrease in *MAP* numbers was observed over ripening, this decrease was independent of lacticin 3147 production. Thus, other strategies will

need to be tested with a view to harnessing the anti-*MAP* activity of this bacteriocin in dairy products.

2.2 Introduction

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is an obligate pathogenic bacterium and is the causative agent of Johne's disease (Cocito *et al.*, 1994), a localized infection of the lower intestine (ileum) which mainly affects ruminants. The resulting lesions from this chronic enteritis are similar to those present in the intestines of patients with Crohn's disease (Davis & Madsen-Bouterse, 2012), consistent with suggestions that this bacterium also contributes to this disease. However, a definite link between *MAP* and Crohn's disease has yet to be established and conflicting research outcomes (Hermon-Taylor, 2002, Momotani *et al.*, 2012) on this subject continues to evoke much debate.

In Ireland it is apparent that the levels of Johne's disease are on the rise. The opening up of the Single European market in 1992 is thought to have contributed to this increase. In 1955, Johne's disease became notifiable (S.I. No. 86 of 1955) although only 92 cases were reported between 1932 and 1992, despite the payment of compensation for confirmed cases. In stark contrast, 232 cases were reported between 1995 and 2002 (Good *et al.*, 2009). As well as the associated economic implications (culling, lower milk and beef yields, veterinary costs), the potential for significant increases in the frequency of *MAP*-containing milk is of concern. Indeed, infected animals excrete the bacterium in large numbers, which contributes greatly to the subsequent infection of calves and the contamination of milk (Fecteau & Whitlock, 2010). This, coupled with *MAP*'s ability to survive outside its host for extended periods of time (Rowe & Grant, 2006) and the possibility of the bacterium surviving pasteurisation, another disputed issue (Lynch *et al.*, 1997, Grant *et al.*, 2002), highlights a route *via* which the pathogen could enter the human food chain.

It is thus a concern that the pathogen has been identified in off-the-shelf dairy products. Out of 357 commercially pasteurized milk samples from Ireland tested, 9.8% were found to be PCR positive for *MAP* (O'Reilly *et al.*, 2004) while, in the US, 2.8% of 702 pasteurised milk samples were found to be culture positive (Ellingson *et al.*, 2005). In the latter case, given the fastidious nature of the strain, this is likely to be a significant under-representation. Given that Irish exports of milk and milk products make a very significant contribution to Irish national GDP, the elimination of any potential zoonotic pathogens from the Irish milk can only be seen as prudent both from a public health and economic perspective.

One potential method to increase the safety of dairy products is through the introduction of additional barriers to aid in the eradication of pathogenic bacteria. One such barrier is the use of bacteriocins (Deegan *et al.*, 2006, Gálvez *et al.*, 2007). Defined as ribosomally synthesised antimicrobial peptides (Cotter *et al.*, 2005), many bacteriocins are produced by lactic acid bacteria. One such strain is *Lactococcus lactis* DPC 3147, which produces the Class 1 bacteriocin, lacticin 3147 (Ryan *et al.*, 1996). *L. lactis* are also the most important industrial starter cultures, both from a biotechnological and economic viewpoint (Bolotin *et al.*, 2001, Fox *et al.*, 2004). The selection of technologically superior strains is based on a number of traits such as rapid lactose metabolism, proteolytic activity, citrate metabolism, bacteriophage resistance, antibiotic resistance, exopolysaccharide (EPS) production and bacteriocin production (Dal Bello *et al.*, 2012). Importantly, many of these traits are plasmid encoded and their self-transmissible nature has been exploited to improve the genotypes of industrial starters in a food grade manner (Mills *et al.*, 2006).

L. lactis DPC 3147 harbours the 60.2 kb plasmid pMRC01. This plasmid carries genes involved in conjugative transfer, phage resistance through abortive infection and the production of and immunity to the lantibiotic, lacticin 3147 (Coakley *et al.*, 1997). Lantibiotics are distinguished by the presence of rare amino acids found within their mature structures, resulting from a number of post-translational modifications (Healy *et al.*, 2010). Two of these, dehydroalanine and dehydrobutyrine, are involved in the formation of the lanthionine bridges, the structures that give these antimicrobials their name. The mode of action of lacticin 3147 is two-fold and involves the synergistic interaction of its two component peptides, Ltn α and Ltn β , leading to the sequestration of essential peptidoglycan precursors and pore formation (McAuliffe *et al.*, 1998, Wiedemann *et al.*, 2006). Like many lantibiotics, lacticin 3147 displays activity in the micro to nano-molar range against many clinically significant pathogens (Piper *et al.*, 2009a, Piper *et al.*, 2009b, Iancu *et al.*, 2012).

There are numerous ways in which lantibiotics can be used in order to enhance the safety of a food(see Deegan *et al.* (2006) for an extensive review on this topic). In this study, an *in situ* method of production by a starter culture in the manufacture of a smear ripened cheese from unpasteurized milk was assessed as a means of controlling the growth of *MAP*. Smear ripened cheeses are characterised by the development of microbial growth on their surfaces during ripening (Brennan *et al.*, 2004). A previous study, by O'Sullivan *et al.* (2006), showed that the introduction of a lacticin 3147 producing *L. lactis* culture to the surface of a smear-ripened cheese led to a 100-fold decrease in *Listeria monocytogenes* numbers. Here, two smear ripened cheeses were manufactured to assess the survival of *MAP* over a 28 day ripening period. The first cheese was made with a lacticin 3147 producing

starter culture while the second was made with a non-bacteriocin producing equivalent and served as a control. It was found that although an approximate 1 log reduction in *MAP* numbers occurred over the ripening period, this reduction occurred regardless of which starter strain was used and, thus, was independent of bacteriocin production.

2.3 Materials and Methods

2.3.1 Bacterial strains and growth conditions

The strains used in this study are listed in Table 2.1. All *L. lactis* strains were grown on/ in M17 agar and broth (Oxoid) supplemented with 0.5% lactose and incubated at 30°C overnight. *MAP* ATCC19698 was grown on Middlebrook 7H9 broth (Becton Dickinson) containing 10% oleic acid-albumin-dextrose-catalase (OADC) supplement (Becton Dickinson) and 0.05% Tween 80. Mycobactine J was added at 2 µg/ ml (Synbiotics Europe) while vancomycin, nalidixic acid and amphotericin B were added at concentrations of 17.5 µg /ml, 17.5 µg/ ml and 12.5 µg/ ml, respectively. Incubation was for 37°C for up to 6 weeks with constant gentle agitation. For enumeration, *MAP* was also grown on Middlebrook 7H10 agar (Becton Dickinson) and Herrold's Egg Yolk agar (HEYA) (Becton Dickinson), with the same supplements and antibiotics as for 7H9 agar. Due to the long incubation times required, all enumerations were carried out in 25 cm³ culture flasks so as to prevent dehydration of the agar (Sarstedt).

2.3.2 Production of smeared cheese from unpasteurised milk

Twenty litres of unpasteurized milk were collected from bulk milk tanks on the Moorepark Farm and refrigerated at 4°C. Samples were analysed to determine total bacterial counts (TBCs) on Milk Plate Count Agar (MPCA), somatic cell counts (SCCs), %fat, %lactose, %protein (all using a MilkoScan 600) and to test for the absence of antibiotics (Delvo test). 10 l of milk were added to two separate vats and heated. At 20°C, 1.5% (v/v) of *L. lactis* DPC3147 (lactacin 3147 producing starter culture) and 1.5% (v/v) of *L. lactis* DPC5399 (non lactacin 3147 producing starter culture) were inoculated into separate vats. At 30°C, 15 ml of the *MAP* strain (~1.0 x

10^9 cfu/ml) was added to each vat to give a final concentration of $\sim 10^5$ cfu/ml and mixed well. Once a pH of 6.55 was reached, 1.6 ml/l of rennet was added to each vat and the milk was left to coagulate for 75 minutes. The curd was cut and allowed to rest for 5 minutes. With constant stirring, both samples were cooked to 36°C over 30 min increasing the temperature by 1°C every 5 min. After cooking, the curd was stirred gently until a pH of 6.0 was reached. At this stage the curd from each vat was added tightly to 6 moulds. These moulds were turned every 30min for the first 3hrs and twice more bringing the total time post-moulding to 5hrs when the moulds were kept at 8°C overnight. The moulds were next brined in 23% sterile NaCl containing 200 $\mu\text{g/l}$ Ca^{2+} for 75 min followed by drainage for 10 min. The cheese was then smeared with Lyrofast SRC1 (Sacco; 1.25 g/250 ml sterile water) and incubated at 13-15°C with 95% relative humidity. The cheeses were turned daily and washed in brine solution every two days. The smear was applied again after day 5. After 2 weeks, the cheeses were moved to an 8°C incubator and allowed to ripen for a further 2 weeks.

2.3.3 Sampling routine

2.3.3.1 MAP enumeration

The raw milk from the bulk tanks was tested to ensure the absence of *MAP*. The cheese was sampled immediately post smearing (T_0) and weekly for 4 weeks during ripening. At each sampling time 5 g of a representative sample (wedge) was homogenised in 2% tri sodium citrate. 100 μl of dilution was added to HEYA and 7H10 agar in 25cm³ culture flask with the relevant selection agents and supplements added. Cetylpyridinium chloride (CPC) (Sigma Chemicals, MO, USA) was added at a concentration of 0.001% to a duplicate of each sample to decrease background contamination. These flasks were incubated at 37°C for 8 weeks.

2.3.3.2 Salt, moisture and pH

All three parameters were determined for both the surface and interior of the cheese using standard methods as described by Lynch *et al.* (1997). Salt concentrations were determined after two weeks of ripening (T2). Measurements were taken to determine moisture and pH immediately post smearing and weekly during ripening.

2.3.4 Detection of lacticin 3147

Well diffusion assays were carried out in LM17 agar seeded (0.5% v/v) with either *L. lactis* HP (lacticin 3147 sensitive) or *L. lactis* HP pMRC01 (lacticin 3147 resistant). 2 g of cheese at T0 (addition of smear) and at week 1, 2, 3 and 4 of ripening was added to 2 ml of 2% tri-sodium citrate, homogenised and centrifuged for 5 min at 13000 rpm. The fat layer was removed with sterile pipette and 50 µl of the supernatant was added to wells in the seeded plates followed by incubation overnight at 30°C. The production of lacticin 3147 was evident through the production of zones against the HP indicator that were not apparent when the HP pMRC01 indicator was used.

2.3.5 Confirmation of *MAP* colonies through PCR

Two separate primer sets (L1 and L2, and AV1 and AV2) (Table 2.2), which target the same *MAP*-specific gene (*IS900*), were employed to confirm the identification of colonies on 7H10 plates. Template DNA was acquired from colonies through IGEPAL denaturation (5 µl incubated 94°C for 10 min). PCR mix (other than primers used, 50 µl total volume) and cycling conditions were the same for both reactions. The PCR mix consisted of 5 µl 10X Buffer for KOD Hot Start DNA Polymerase, 3 µl 25mM MgSO₄, 5 µl of 2 mM dNTPs, 2.5 µl 0.2 µM forward and reverse primer, 5 µl of template from previous reaction, 1 µl of KOD Hot Start DNA

Polymerase (1U μ l) (Novagen) and 26 μ l of H₂O. Initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 20 sec, 58°C for 10 sec and 72°C for 15 sec with a final extension of 72°C for 5 min.

2.3.6 Molecular Quantification using RT-PCR

2.3.6.1 Sample preparation prior to DNA extraction

Duplicate 5g samples of cheese from each sample period were diluted 1:10 in cheese diluent composed of 0.5% (wt/vol) sodium chloride, 1% (wt/vol) Casitone (Difco), and 2% (wt/vol) sodium citrate. This mixture was next stomached at 260 rpm for 4 min. The samples were incubated for hr at 37°C shaking at 90°C followed by stomaching at the same conditions as above. This mix was then centrifuged for 15 min at 4000 rpm. The supernatant was removed and the remaining pelleted resuspended in 10 ml of phosphate buffered saline.

2.3.6.2 DNA Extraction

DNA extraction was achieved using an Adiapure paraTB Milk kit (Adiagene). The procedure was carried out as stated in manufacturer's instructions. In brief, *MAP* DNA was separated through the specific capture of *MAP* cells on magnetic beads with subsequent grinding to destroy the cell wall.

2.3.6.3 Real-Time-PCR

This method used was based on that developed by Rodriguez-Lazaro *et al.* (2005). The target area for the RT-PCR was the IS900 insertion sequence in the *MAP* genome. Applied Biosystems® 7500 Fast Real-Time PCR System was used for the analysis. Taqman® Universal PCR MasterMix (Applied Biosystems®) was used as per manufacturer's specifications. To ensure fluorescence was not being inhibited, a

Taqman IPC (Applied Cat. No.43083223) was included. The primers and probe used are listed on Table 2.2.

2.4 Results

For this study, two separate cheese trials were carried with the aim of determining if *MAP* could survive the process involved in the production of a smear ripened cheese from raw milk. The second goal was to determine if the presence of a lactacin 3147-producing starter culture could provide an additional means of eradicating this pathogen from the human food chain. Finally, a comparison was made between the 'gold standard' plate based enumeration method with a molecular quantification technique to quantify *MAP* levels.

As the cheese was made from raw unpasteurised milk, a high microbial load was to be expected both after manufacture and during ripening. Added to this was the large consortium of bacteria arising from the smear that was applied post production. These features and the extremely fastidious nature of the pathogen meant that isolation of *MAP* at low levels was impractical. Thus, it was decided that the starting inoculum for the *MAP* strain in the raw milk was to be approximately 10^5 cfu/ml.

The raw milk for this trial was obtained from the bulk milk tank from the Teagasc Moorepark farm. Table 2.3 displays the percentage fat, the percentage lactose and the percentage protein - all of which were at acceptable limits. The total bacterial count and the somatic cell count were also below stated limits (Regulation (EC) No. 853/2004) and no antibiotic residues were detected. The smear ripened cheese make procedure was carried out as outlined in the materials and methods section. Along with the enumeration of *MAP* during the ripening procedure, the percentage moisture and pH was recorded at Day 0 (addition of the smear), and weekly for a period of four weeks.

Figure 2.1, panels A-E shows the development of the smear over the ripening period. For this study a fast-growing commercial blend was used, Lyrofast SRC1 (Sacco), a blend which consists of specifically selected strains of *Debaryomyces hansenii*, *Staphylococcus xylosus*, *Arthrobacter globiformis*, *Brevibacterium linens* and *Geotrichum candidum*. The low starting pH favours the growth of *D. hansenii* and *G. candidum* which in turn increases the pH leading to conditions that favour the growth of the remaining members of the consortium. During this process the smear develops on the surface resulting in a softening of the cheese through the proteolytic and lipolytic action of the smear bacteria. As the smear develops a yellow colour appears on the surface.

The percentage moisture, for both the surface and the interior of the cheese, was monitored (Figure 2.2). Unlike cheeses such as cheddar or Gouda, which are either vacuum-packed in plastic or have a wax coat applied during ripening, a smear type cheese is ripened without any physical protection. In order to protect the cheese from moisture loss, ripening took place within a humidity controlled chamber. While some variation was noted for the % moisture on the surface of both cheeses at day 28, moisture levels which ranged from 44% up to 60% were within the accepted guidelines for a smear type cheese.

As is normal for a smear type cheese, the pH (Figure 2.3) on the surface increased from pH 5.1 on Day 0 to over pH 7.0 at the end of ripening for both the control and lacticin 3147 containing cheese. This increase corresponds to the development of the smear. A more subtle rise in pH was seen for the interior of both cheeses with a difference of 0.2 pH units observed. Both the percentage moisture and pH results shown here are averages of both trials.

Unlike Cheddar, which is dry salted before pressing, smear cheeses are brine salted after molding. As time is needed for the migration of the salt from exterior to interior, and to eventually reach an equilibrium, the percentage salt was measured after 2 weeks ripening. Samples were taken from the surface, the interior and midway. The mean of these three values was averaged from both trials and the standard deviation determined. The percentage salt for the control cheese was 1.4 with a SD of 0.106 while the lacticin 3147 containing cheese was 1.5 with a SD of 0.213. Both figures fall within acceptable norms for this type of cheese.

Well diffusion assays were employed to ensure that lacticin 3147 was produced at all stages during ripening. *L. lactis* HP was used as a lacticin 3147 sensitive indicator while a derivative of HP that contains a plasmid, pMRC01, encoding for immunity to the lantibiotic was also used to ensure that any inhibition was due to the lacticin 3147 specifically and not another inhibitor in the cheese matrix. In all cases, no lacticin 3147 production was detected in the control cheese while a zone of inhibition was observed in the test cheese throughout ripening (data not shown). It was thus apparent that the *MAP* cells were exposed to lacticin 3147 from manufacturing to ripening.

Survival of *MAP* was monitored over the 28 day ripening period by plating on HEYE and 7H10 (Figure 2.4). The initial 1:10 dilution was made in 2% tri-sodium citrate with subsequent dilutions made in maximum recovery diluent (MRD). Each sample was weighed twice. In order to reduce background flora, 0.001% CPC was added to one while the second was plated without addition of the antiseptic. At T0, *MAP* cells were detected at $\sim 10^5$ cfu/g in both trials and in both the control and lacticin 3147 cheeses. An approximate 1 log reduction was observed for both the control and the test cheese over the first two weeks of ripening with this figure

remaining constant at the end of week three. By the end of week 4, the levels of *MAP* showed a slight increase. Both the control and the test cheese when treated with CPC showed a more constant decrease in cell numbers with a log reduction of 2.4 observed for each. HEYM was found to be unsuitable for the recovery of *MAP* from raw milk due to the high levels of background contamination present on the plates. Therefore all counts were from the 7H10 plates. No difference was observed in *MAP* levels between cheeses containing the lacticin 3147 producing starter culture and the control, either with or without the addition of CPC. All presumptive *MAP* colonies were confirmed as *MAP* through PCR with the use of two IS900 primer sets, L1& L2 and AV1 & AV2 (Bull *et al.*, 2003).

Molecular quantification of the levels of *MAP* in the cheese samples was completed using real-time PCR so as to ascertain whether this was a valid method for future studies. The resulting Ct values (Table 2.4) indicate a good replication of the trend observed for the plate based enumeration in that a less than 1 log reduction was observed over the ripening period and no discernible difference in numbers was apparent between cheese containing the lacticin 3147 producing starter culture and that produced with the non-bacteriocin producing equivalent.

2.5 Discussion

Members of the family Mycobacteriaceae include many slow-growing pathogenic species such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the etiological agents of human tuberculosis and leprosy, respectively. Two important animal pathogens also belong to this family; *Mycobacterium bovis*, the causative agent of bovine tuberculosis, and *Mycobacterium paratuberculosis*, the causative agent of Johne's disease. It is the latter bacterium which is the focal point of this study.

Johne's disease has been detected on every continent (Rowe & Grant, 2006) and, due to difficulties in its detection during a sub-clinical period of between 2 to 5 years (Stabel, 1998), treatment of the condition is challenging. The clinical period of infection manifests as a chronic and intermittent diarrhoea leading to contamination of the environment and colostrum, with increased herd infection (Cocito *et al.*, 1994). Infected cattle become increasingly emaciated and die of dehydration and severe cachexia. Coupling this with *MAP*'s innate resistance to chlorine (Whan *et al.*, 2001) and processes such as temperature treatments (Grant *et al.*, 2002) afforded to it by its waxy cell wall, contaminated milk provides a realistic route for the introduction of the bacterium into the human food chain.

Three questions were addressed in this study. Firstly, does *MAP* survive manufacture and ripening of a smear ripened cheese made from unpasteurized milk? Cheeses of this variety are high risk products with respect to contamination by pathogens due the risks associated with the use of unpasteurised milk and technological factors such as their high moisture content, low salt concentrations and short-ripening times. Secondly, does the bacteriocin producing strain *L. lactis* DPC

3147, when used as a starter culture in the same process, impact on the survival of the pathogen? Thirdly, we compared the gold standard method of *MAP* enumeration, i.e. culturing on solid media, with a real-time PCR technique targeting the IS900 insertion sequence (McFadden *et al.*, 1987, Green *et al.*, 1989), which is present at 14-18 copies per genome.

Lantibiotics such as nisin and lacticin 3147 have previously demonstrated effectiveness against a number of clinically significant mycobacteria (Carroll *et al.*, 2010). Indeed in that previous study, a purified form of lacticin 3147 displayed particularly potent activity against *Mycobacterium tuberculosis* H37Ra, *Mycobacterium kansasii* CIT11/06 and *MAP* ATCC19698. This activity provided the rationale for employing a lacticin 3147 producing starter for this cheese trial.

Plating on selective agar, without CPC treatment, (Figure 2.4) revealed that viable counts of *MAP* from both the control and lacticin 3147 producing starter-containing cheeses decreased less than 10-fold after four weeks of ripening. Although lacticin 3147 was produced throughout the ripening period, as demonstrated by well diffusion assays, it had no effect on *MAP* numbers. Current culture methods do not allow the enumeration of low numbers of *MAP* in the presence of high background microflora found in smear ripened raw milk cheese. In this study an artificially high inoculum was used for these reasons. It may be that bacteriocin producing cultures would provide significantly improved killing of *MAP* if the levels of the pathogen are within the normal range for naturally contaminated milk. As the % moisture, pH and appearance of both the control and the lacticin 3147 containing cheeses were all within the accepted norms, it could be concluded that the presence of lacticin 3147 in the cheese matrix, which was present right through ripening, did not impact on the bacterial constituent of the smear.

When samples were treated with CPC prior to plating, a more dramatic decrease in cell numbers was observed. For both the control and the test cheese, a greater than 2 log reduction was observed. This decrease is likely due to the inability of *MAP* cells that are sub-lethally injured as a result of the ripening process to survive CPC treatment. The similarity in *MAP* survival levels across the control and the test cheeses again highlighted that bacteriocin production did provide enhanced protection against the pathogen.

RT-PCR may be a valuable tool to confirm the viability of *MAP* in a complex matrix, such as smear ripened raw milk cheese, where the bacterial load is extremely high and the flora quite diverse. In this study, RT-PCR results confirmed the conclusions reached from plate enumeration, including the observation that lacticin 3147 producing cultures did not offer added protection through inhibition of the *MAP* cultures during cheese ripening in raw milk smear ripened cheese. As noted previously, due to sensitivity limits, the starting inoculum was high, which may have impacted on the effectiveness of the bacteriocin. As the sensitivity afforded by this molecular quantification is lower than that of plate counts, future studies could assess the effectiveness of bacteriocins at more realistic levels of *MAP* contamination.

Although not classed as a zoonotic agent, *MAP* has often been linked with Crohn's disease, an inflammatory bowel disorder of global importance. In this study, a possible route of infection was assessed, i.e. smear-ripened cheese from raw milk. Even with a bacteriocin producing starter culture, this study indicates that the bacterium would survive manufacture and ripening. Interestingly, lacticin 3147 did not impair the normal cheese flora and therefore could be employed in future trials, with RT-PCR-based detection, using a *MAP* inoculum more reflective of normal

contamination. However, it is clear from this study that raw milk from animals shedding high numbers of *MAP* cells should not be used for the manufacture of smear type cheese.

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2.8 Tables and Figures

Table 2.1 Strains used in this study

Strains	Characteristic	Reference/Source
<i>L. lactis</i> DPC3147	Natural lacticin 3147 producing starter culture	<i>Ryan et al. (1996)</i>
<i>L. lactis</i> DPC5399	Bac ⁻ Isogenic strain of DPC3147 starter culture	DPC Culture Collection
<i>L. lactis</i> HP	Lacticin 3147 sensitive indicator	DPC Culture Collection
<i>L. lactis</i> HP pMRC01	Lacticin 3147 resistant indicator	DPC Culture Collection
<i>MAP</i> ATCC19698	Target organism	ATCC

DPC: Dairy Products Research Centre, ATCC: American Type Culture Collection

Table 2.2 Oligonucleotides used in this study

Oligonucleotide	Target gene	Sequence	Reference
L1	IS900	5'-CTTTCTTGAAGGGTGTTCCGG-3'	(Bull <i>et al.</i> , 2003)
L2	IS900	5'-ACGTGACCTCGCCTCCAT-3'	
AV1	IS900	5'-ATGTGGTGGCTGTGTTGGATGG-3'	
AV2	IS900	5'-CCGCCGCAATCAACTCCAG-3'	
IS900QFForward	IS900	5'-CCGGTAAGGCCGACCATTA-3'	(Rodriguez-Lazaro <i>et al.</i> , 2005)
IS900QR Reverse	IS900	5'-ACCCGCTGCGAGAGCA-3'	
IS900QP Probe	IS900	5'-FAM-CATGGTTATTAACGACGACGCGCAGC-TAMR -3'	

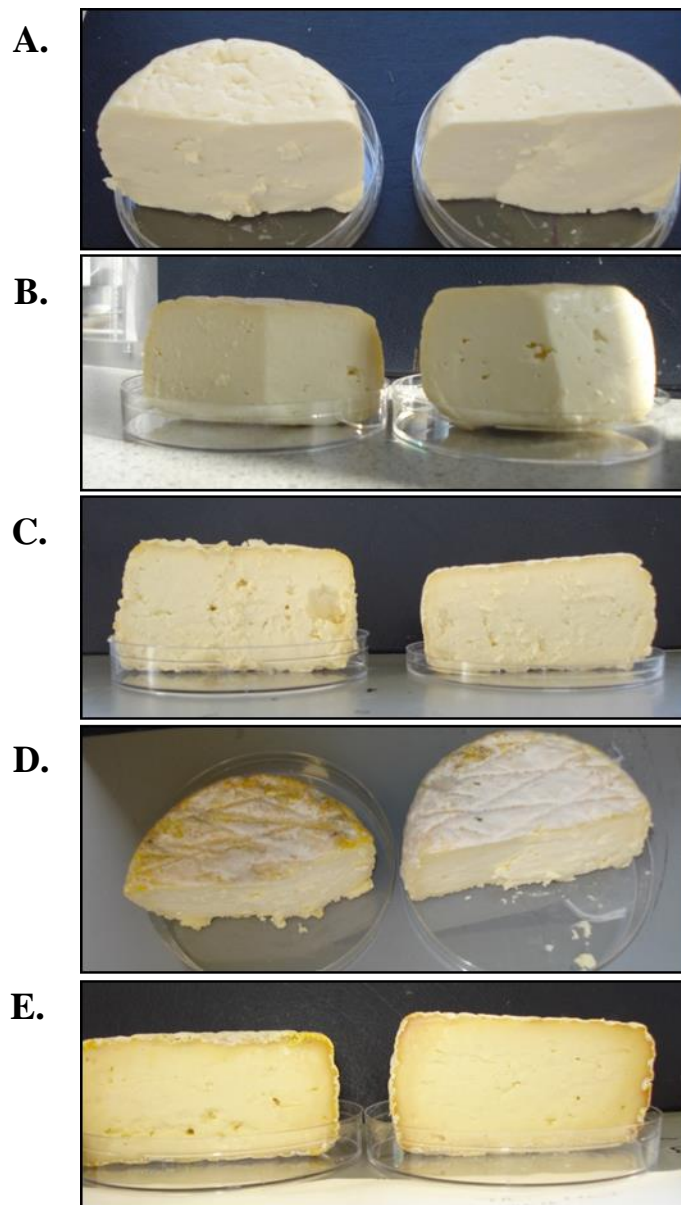
Table 2.3 Raw Milk Analysis

Analysis	Cheese Trail 1	Cheese Trial 2
Total bacterial count	9.9×10^3 cfu /ml	4.4×10^3 cfu/ml
Somatic cell count	3.4×10^5 cfu/ml	2.8×10^5 cfu/ml
% Fat	4.53	4.30
% Lactose	4.46	4.71
% Protein	3.16	3.24
Antibiotic residues	Absent	Absent

Table 2.4 RT-PCR Results

Sample Reference	Ct Value
Trail 1 Day 0 Control	18.0000
Trail 1 Day 0 Lacticin 3147 Producer	17.4656
Trail 1 Week 1 Control	17.2108
Trail 1 Week 1 Lacticin 3147 Producer	16.9647
Trail 1 Week 2 Control	16.9362
Trail 1 Week 2 Lacticin 3147 Producer	16.6056
Trail 1 Week 3 Control	17.1505
Trail 1 Week 3 Lacticin 3147 Producer	16.5786
Trail 1 Week 4 Control	17.5554
Trail 1 Week 4 Lacticin 3147 Producer	17.3316
Trail 2 Day 0 Control	17.2042
Trail 2 Day 0 Lacticin 3147 Producer	18.0386
Trail 2 Week 1 Control	16.2401
Trail 2 Week 1 Lacticin 3147 Producer	16.4913
Trail 2 Week 2 Control	16.1759
Trail 2 Week 2 Lacticin 3147 Producer	16.5469
Trail 2 Week 3 Control	15.9728
Trail 2 Week 3 Lacticin 3147 Producer	16.1388
Trail 2 Week 4 Control	19.6297
Trail 2 Week 4 Lacticin 3147 Producer	16.3408

Figure 2.1 Production of the smear on the surface of the cheese



The growth of the smear from appliance (panel A.) and its growth over the ripening period of the cheese; panel B. after 1 week, panel C. after 2 weeks, panel D. after 3 weeks and panel E. after 4 weeks.

Figure 2.2 % Moisture during ripening period

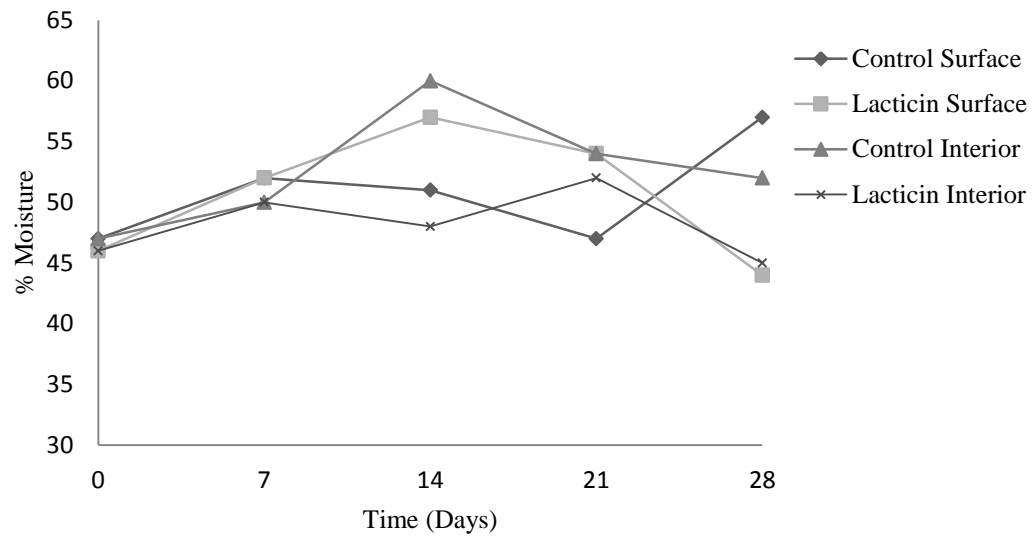


Figure 2.3 pH change during ripening period

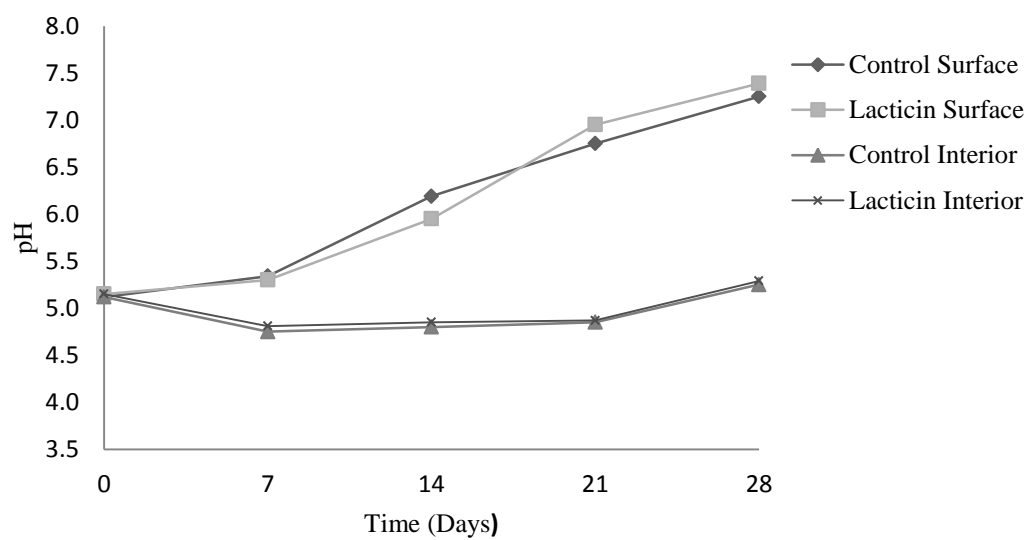
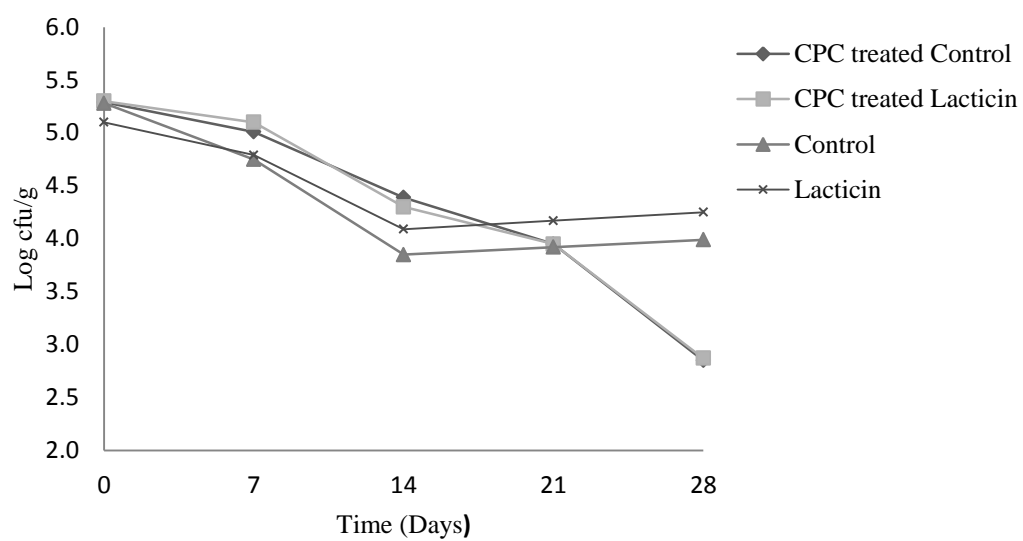


Figure 2.4 *MAP* enumerations over 28 day ripening period



Survival of *MAP* ATCC19698 in a raw milk smear ripened cheeses in the presence and absence of a lacticin 3147-producing starter culture. CPC - Cetylpyridinium chloride

Chapter III

Intensive mutagenesis of the Nisin hinge leads to the rational design of enhanced derivatives

Brian Healy, Des Field, Paula M O'Connor, Colin Hill, Paul D. Cotter and R. Paul Ross

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3.1 Abstract

Nisin A is the most extensively studied lantibiotic and has been used as a preservative by the food industry since 1953. This 34 amino acid peptide contains three dehydrated amino acids and five thioether rings. These rings, resulting from one lanthionine and four methyllanthionine bridges, confer the peptide with its unique structure. Nisin A has two mechanisms of action, with the N-terminal domain of the peptide inhibiting cell wall synthesis through lipid II binding and the C-terminal domain responsible for pore-formation. The focus of this study is the three amino acid ‘hinge’ region (N 20, M 21 and K 22) which separates these two domains and allows for conformational flexibility.

As all lantibiotics are gene encoded, novel variants can be generated through manipulation of the corresponding gene. A number of derivatives in which the hinge region was altered have previously been shown to possess enhanced antimicrobial activity. Here we take this approach further by employing simultaneous, indiscriminate site-saturation mutagenesis of all three hinge residues to create a novel bank of nisin derivative producers. Screening of this bank revealed that producers of peptides with hinge regions consisting of AAK, NAI and SLS displayed enhanced bioactivity against a variety of targets. These and other results suggested a preference for small, chiral amino acids within the hinge region, leading to the design and creation of producers of peptides with hinges consisting of AAA and SAA. These producers, and the corresponding peptides, exhibited enhanced bioactivity against *Lactococcus lactis* HP, *Streptococcus agalactiae* ATCC 13813, *Mycobacterium smegmatis* MC2155 and *Staphylococcus aureus* RF122 and thus represent the first example of nisin derivatives that possess enhanced activity as a consequence of rational design.

3.2 Introduction

Bacteriocins are small, bacterially produced, ribosomally synthesized peptides that are active against other bacteria and against which the producer has a specific immunity mechanism (Cotter *et al.*, 2005, Cotter *et al.*, 2013). The bacteriocins can be subdivided on the basis of their structure with Class 1 consisting of peptides that have undergone post-translational modification (Rea *et al.*, 2011). Of these, the lantibiotics have been the focus of particular attention (Chatterjee, 2005, Bierbaum & Sahl, 2009). The name of these bacteriocins reflects their structure; ‘**L**anthionine-containing **antibiotics**’, where lanthionines/ β -methyllanthionines are unusual residues that are formed between cysteines and neighbouring dehydrated serines (dehydroalanines) or threonines (dehydrobutyrines), respectively (Bierbaum & Sahl, 2009).

The lantibiotic Nisin A (Fig. 3.1) has been used a commercial food additive since 1953 and has been approved for use in food by the FAO, WHO, EU and the USFDA (Delves-Broughton *et al.*, 1996, Cotter *et al.*, 2005). Unsurprisingly, Nisin A is by far the most extensively studied lantibiotic (Delves-Broughton *et al.*, 1996, Cotter *et al.*, 2005, Lubelski *et al.*, 2008). In addition, the gene encoded nature of nisin, and indeed other lantibiotics, can be exploited through engineering to even further enhance its activity and/ or investigate structure-function relationships. Such engineering can be carried out *in vivo* (manipulation of the producer or heterologous expression of the genes in another host) or *in vitro* (using purified components of the biosynthetic machinery) (Rink *et al.*, 2005, Field *et al.*, 2008, Healy *et al.*, 2010, Knerr & van der Donk, 2012).

The *in vivo* engineering of nisin through the replacement of specific amino acids commenced in 1992 (Kuipers *et al.*, 1992) and a number of derivatives were identified that have been of considerable value with respect to revealing the fundamentals of nisin biology (Cotter *et al.*, 2005, Field *et al.*, 2010). More recently, derivatives have been identified with academic and potential commercial value that display enhanced activity against pathogenic bacteria (Yuan *et al.*, 2004, Field *et al.*, 2008, Field *et al.*, 2012, Molloy *et al.*, 2012, Rouse *et al.*, 2012, Molloy *et al.*, 2013). The majority of these enhanced derivatives differ with respect to the amino acids found in the 3 amino acid 'hinge' region of the peptide. This region is thought to be key with respect to linking the two functional domains of the nisin peptide and providing conformational flexibility between these regions (Breukink *et al.*, 1997, Wiedemann *et al.*, 2001). Nisin inhibits cell wall synthesis through the formation of a complex with lipid II, an essential precursor of peptidoglycan synthesis (Brotz *et al.*, 1998, Breukink *et al.*, 1999), with the N-terminus region being responsible for this binding (Hsu *et al.*, 2004). The hinge links this domain with the C-terminal end of the peptide, which is responsible for a second mechanism of antimicrobial activity, which involves permeabilisation of the cell membrane. The first indication that mutagenesis of the hinge could bring about beneficial consequences was provided by Yuan and co-workers (2004) who established that two derivatives of nisin Z (N20K and M21K), exhibited enhanced activity against Gram-negative (*Shigella*, *Pseudomonas* and *Salmonella*), but not Gram-positive, pathogens. This was followed by the identification of hinge derivatives, such as N20P, M21V, K22S and K22T (Field *et al.*, 2008), with enhanced activity against Gram-positive pathogens (*Streptococcus agalactiae*, *Staphylococcus aureus* and *Listeria monocytogenes*). Further studies have emphasised the enhanced potency of one of

these variants, nisin V (M21V) (Field *et al.*, 2010), against a broad variety of drug-resistant pathogens and have identified other hinge derivatives, such as that containing the residues SVA, that exhibit enhanced activity in complex matrices (Rouse *et al.*, 2012).

The hinge derivatives that have been studied to date have resulted from strategies in which one or two of the hinge residues have been manipulated. Here we go a step further through the randomisation of all three hinge residues simultaneously. Screening of a bank of producers of such derivatives revealed a pattern whereby many producers of derivatives containing small chiral amino acids within the hinge displayed enhanced bioactivity. This prompted the rational design of additional derivatives not identified from the random bank, peptides containing AAA and SAA hinges, which were particularly notable with respect to the extent to which bioactivity was enhanced, relative to the wild-type producer.

3.3 Materials and Methods

3.3.1 Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table 3.1 *Lactococcus lactis* cultures were grown in M17 broth or agar (1.5%) (Oxoid) supplemented with 0.5% glucose at 30°C. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or agar with continuous shaking at 37°C. Where necessary, chloramphenicol was used at 10 µg/ml for *L. lactis* and *E. coli*. *Staphylococcus aureus* RF122 was grown in Brain Heart Infusion (BHI) broth or agar (Oxoid) at 37°C. *Streptococcus agalactiae* ATCC 13813 was grown in Tryptic Soy Broth (TSB) (Merck) or TS agar supplemented with Yeast Extract (YE) (Oxoid) at a concentration of 0.6% at 37°C. *Mycobacterium smegmatis* MC2155 was grown on Middlebrook 7H9 broth (BD) or Middlebrook 7H10 agar (BD) supplemented with 0.05% tween 80 and 2% glycerol at 37°C.

3.3.2 Site-saturation mutagenesis of the nisin A ‘hinge’ region

The complete randomisation of the ‘hinge’ amino acids was achieved by PCR using the template pDF05 (pCI372–*nisA*) and primers NisAXXXHingeFor and NisAXXXHingeRev (Table 3.2). The template DNA was extracted using a High Pure Plasmid Isolation Kit (Roche) from dam⁺ *E. coli* Top10 (Invitrogen) to ensure its methylation. PCR amplification was performed in 50µl volumes with 1 ng per 50 µl of template, 2 units of Phusion High Fidelity DNA Polymerase (Finnzymes), approximately 0.5 ng of template, 1x HF buffer, 200 µM dNTPs and 0.5 µM of the relevant oligonucleotides. Cycling conditions were as follows: 98°C for 30 secs, 55°C for 15 secs, 72°C for 3.5 mins for 40 cycles followed by final extension for 10 mins. Samples were PCR cleaned using GeneJet PCR Purification Kit (Thermo

Scientific) followed by *DpnI* treatment (Stratagene) at 37°C for 3 hours. 2.5 µl of mutated pCI372-NisA was added to a vial of Top 10 Chemically Competent *Escherichia coli* (Invitrogen). These transformants were pooled, plasmids extracted using a High Pure Plasmid Isolation Kit (Roche) and transformed into electro-competent *L. lactis* ssp. *cremoris* NZ9800Δ*nisA* and selected for on GM17 Cm¹⁰ agar within Q-Trays. Resultant colonies were picked and added to GM17 within Genetix 96-well plates (Genetix X6011), incubated overnight at 30°C before storage at -80°C in 44% glycerol (Sigma).

3.3.3 Site-directed mutagenesis of the nisin A ‘hinge’ region

The creation of targeted changes was also facilitated using a PCR-based approach, using oligonucleotides (Table 3.2) and a suitable pDF05 based template (one which most closely resembles the desired change). Amplification was through a 50µl PCR reaction containing 0.02U/µl KOD Hot-Start Polymerase (Novagen), 1.5 mM MgSO₄, 0.2 mM of each dNTP, 0.3 µM of both oligonucleotides and approximately 10 ng of template. Cycling conditions were as described above. Engineered plasmids were introduced into *L. lactis* NZ9800Δ*nisA* via *E. coli* Top10 as described above.

3.3.4 Deferred antagonism assays

Deferred antagonism agar-based assays were employed to assess the bioactivity of nisin derivative-producing strains (Rouse *et al.*, 2012). Briefly, the *L. lactis* producers were ‘spotted’ (approximately 3 µl) onto GM17 agar and incubated for 16 hours at 30°C. In the case of *M. smegmatis*, the *L. lactis* producers were incubated for 40 hours. Growth media (0.75% agar) appropriate for growth of the individual target was seeded (0.5%) and poured over the *L. lactis* producers followed by further incubation at conditions suitable for the indicator. Enhancement in bioactivity was

indicated by increased zone of inhibition relative to that generated by the wild-type producer.

3.3.5 Agar well diffusion assays

50 µl volumes of purified peptide were added to wells bored in the appropriate agar-containing media (1.5% agar, 0.75% for *M. smegmatis*) seeded with 0.5% of a 16 hour culture (40 hour culture for *M. smegmatis*) of the indicator of interest. Plates were incubated for 16 hours (40 hours for *M. smegmatis*) and bioactivity assessed on the basis of the size of the zone of inhibition.

3.3.6 Identification of nisin A derivatives

Colony mass spectrometric analysis was carried out on colonies exhibiting enhanced bioactivity using an Axima CFR plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) and analyzed in positive-ion reflectron mode as previously described (Field *et al.*, 2008). The changes to the *nisA* genes within the corresponding pDF05 derivatives were established through DNA sequencing (MWG, Biotech, Germany). The sequences for AAA, AAK, NAI, SAA and SLS were deposited in GenBank under the accession numbers KF664587, KF664588, KF664589, KF664590 and KF664591, respectively.

3.3.7 Purification of nisin A and derivatives thereof

2 litres of Tryptone Yeast (TY) broth were incubated for 20 hours with 20 ml of an overnight culture of producing strain. This culture was centrifuged for 20 minutes @ 8630g. The supernatant was decanted and passed through 60g of pre equilibrated Amberlite XAD16 beads (Sigma-Aldrich). The beads were washed with 500ml 30% ethanol and eluted with 500 ml 70% isopropanol (IPA) (Fisher) 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich). Concomitantly, the cell pellets were resuspended in

300 ml of 70% IPA 0.1% TFA and stirred at room temperature for 3 hours followed by centrifugation. This cell supernatant was combined with that referred to above and concentrated through rotary-evaporation (Buchi, Switzerland) to approximately 250ml. After the pH was adjusted to 4.5 further concentration was achieved through the use of a Phenomenex SPE C-18 column to a final volume of 60 ml. 7 ml of this sample was concentrated, through rotary evaporation, to 2 ml and purified through HPLC using a Phenomenex C12 Reverse-Phase (RP) HPLC column (Jupiter 4 μ proteo 90 Å, 250 X 10.0 mm, 4 μ m). To facilitate this, a gradient of 30-50% acetonitrile (Fisher) containing 0.1% TFA was developed. The relevant fractions were collected and pooled, subjected to rotary-evaporation to remove acetonitrile and freeze-dried (LABCONCO). The purified peptides were subjected to MALDI-ToF Mass Spectrometric analysis to confirm their purity before use.

3.3.8 Minimum Inhibitory Concentration (MIC) Assays

Minimum inhibitory concentration assays were carried out in triplicate using 96-well plates (Sarstedt) pretreated with bovine serum albumin (BSA) as previously described (Wiedemann *et al.*, 2006). Wild-type nisin A and nisin derivatives were adjusted to a 500 nM (when using *L. lactis* as a target) or 7.5 μ M (all other indicators) starting concentration and 2-fold serial dilutions of each peptide were carried out. An overnight of the target strain was subcultured and incubated to an OD_{600nm} of 0.5 before being diluted to give a final inoculum of 10^5 cfu/ml in 200 μ l. The plates were incubated at an appropriate temperature and inspected after 16 hours. The MIC was determined as the lowest concentration at which no growth was visible.

3.4 Results

3.4.1 Creation of a bank of producers of randomised hinge derivative

In order to fully exploit the potential of the nisin ‘hinge’ region (N20-M21-K22) to generate enhanced derivatives, it was decided to undertake a complete randomisation of this area. Using NNK scanning of the nisin A structural gene (*nisA*), a large bank of *L. lactis* NZ9800 pCI372*nisA* (pDF05) hinge variants were produced. In order to obtain full coverage with a 95% confidence limit using NNK scanning on three positions, a bank of 341,601 variants would have to be screened (Nov, 2012). Creating and screening a bank of this size against four indicators would not have been feasible and thus a more practical bank of 12,000 variants was produced in order to increase the likelihood of finding interesting candidates in a short/ medium time frame.

3.4.2 Identification of nisin derivative producers with enhanced bioactivity

The bank of producers of randomised hinge derivatives was screened using deferred antagonism agar diffusion assays to identify producers that display enhanced bioactivity. The term bioactivity, as used here and elsewhere (Field *et al.*, 2008, Rouse *et al.*, 2012, Molloy *et al.*, 2013), reflects the overall activity of producer strains and does not discriminate between effects due to increased/ decreased specific activity, altered peptide production levels, or effects on other physico-chemical properties such as diffusion in agar. Enhanced bioactivity was characterised by zones of clearing greater than that generated by the corresponding nisin A producing control against *Lactococcus lactis* HP, *Streptococcus agalactiae* ATCC 13813, *Mycobacterium smegmatis* MC2155 or *Staphylococcus aureus*

RF122. From this screen 63 potentially enhanced producers were selected for further investigation and, after DNA sequencing, it was established that these corresponded to 23 unique mutants (Table 3.3). These 23 derivatives contained hinge regions consisting of combinations of 12 distinct amino acids (Table 3.4), none of which were aromatic or negatively charged in nature. Of these 12, alanine was most common (22%), followed by serine (16%) and glutamine (12%). To exclude that a bias or over-presentation of specific residues had occurred, and to insure the overall randomisation of the bank, 20 clones were chosen randomly and their hinge region was sequenced. The results are presented in Table 3.5 and establish that alanine and serine are not considerably over-represented within the bank. From the agar diffusion assays it was apparent that three producers consistently produced large zones of inhibition and these were selected for further investigation. DNA sequencing and mass spectrometric analysis revealed that the derivatives produced by these strains contained hinge regions consisting of AAK, SLS and NAI (Table 3.6). Assays with four target microorganisms established that the producer of the AAK-containing derivative exhibited enhanced bioactivity against *L. lactis* HP, *S. aureus* RF122 and *M. smegmatis* MC2155, the producer of the SLS-containing derivative exhibited enhanced bioactivity against *L. lactis* HP and *M. smegmatis* MC2155 and the producer of the NAI-containing derivative exhibited enhanced bioactivity against *L. lactis* HP, *S. agalactiae* ATCC 13813 and *M. smegmatis* MC2155. This enhancement could potentially be as a result of enhanced production, enhanced specific activity or some other enhancement with respect to the attributes of the peptide. To investigate these various possibilities, the three peptides produced by these strains (and wild-type nisin A) were purified through HPLC and broth-based specific activity and agar diffusion assays were performed (Table 3.7). Against *L.*

lactis HP, all three bioengineered peptides displayed enhanced activity, relative to equal concentrations of nisin A, when assessed through agar diffusion assays. However, this was not due to enhanced specific activity as broth-based MIC assays revealed that the activity of the NAI-containing peptide was equal, and those of the AAK- and SLS-containing peptides were reduced, relative to that of nisin A against this target (Table 3.7). All of the nisin derivatives displayed specific activity in broth which was reduced relative that of the wild-type peptide against *S. aureus* RF122, yet the AAK- and NAI-containing peptides exhibited enhanced activity when assessed through agar diffusion assays, with the enhanced activity of the AAK-containing peptide being most significant (Table 3.7). When tested against *S. agalactiae* ATCC 13813, the NAI-containing peptide displayed significantly enhanced activity against this target in agar diffusion assays and in broth based specific activity assays. Regardless of assay, neither the AAK- nor the SLS-containing peptides exhibited enhanced activity against *S. agalactiae* ATCC 13813 (Table 3.7). *M. smegmatis* MC2155 was also included as an indicator as *M. smegmatis* is frequently used as a model microorganism/ substitute for slow-growing, pathogenic mycobacteria. The AAK-containing peptide and the SLS-containing peptides both showed significant enhanced activity against this target in agar diffusion assays (Table 3.7), but the aggregative nature of MC2155 in broth precluded the generation of consistent MIC data from broth-based studies.

3.4.3 Rational design of nisin derivatives with enhanced bioactivity

The amino acid composition of nisin hinges within strains exhibiting enhanced bioactivity revealed some trends. All ‘improved’ hinge regions had a mass less than that of the wild-type, alanines were frequently identified at each position and, on a

number of occasions, multiple alanines were present. Based on these observations, it was postulated that a nisin derivative with a hinge consisting of AAA could potentially display enhanced properties. The presence of a serine, particularly at position 20, in the nisin hinge of other strains displaying enhanced bioactivity has also been noted, including hinges consisting of SMT and SLS from this study and SVA from Rouse *et al.* (2012). On the basis of this observation, a nisin derivative containing a SAA hinge was also created. To further test the theory that nisin derivatives with a hinge consisting of small amino acids may exhibit enhanced features, site-directed mutagenesis was also used to create a hinge derivative consisting of glycine residues only. In this last case, the producer of the GGG-containing hinge did not exhibit antimicrobial activity. It would thus seem that a lack of chirality within the hinge is a negative feature.

In contrast, the respective producers of the AAA- and SAA-containing peptides displayed enhanced bioactivity against each of the four strains tested and again were purified for specific activity assays (Tables 3.6 and 3.7). Both the AAA- and SAA-containing peptides showed significantly enhanced specific activity relative to nisin A against all four indicators in agar diffusion assays. In each case, this enhancement was more significant in the case of the AAA-containing peptide. In contrast, in broth based specific activity assays the bioengineered peptides exhibited activity that was equal or reduced relative to that of the wild-type peptide, with the exception that the AAA-containing peptide showed a two-fold improvement relative to the natural peptide against *S. agalactiae* ATCC 13813. Thus, while in many cases it would appear that enhanced bioactivity is attributable to enhanced diffusion through complex media, a phenomenon previously reported by Rouse *et al.* (2012), this

result establishes that antimicrobial potency can also be a contributory factor with respect to some targets.

3.5 Discussion

In this study a large bank of derivatives of the lantibiotic nisin was generated in which all three hinge residues were simultaneously randomised. To facilitate the relatively rapid screening of such a large number of derivative-producing strains, the deferred antagonism assay was employed. This approach allows for the identification of variants with enhanced bioactivity and can be supported by further assays to determine if this enhanced bioactivity is attributable to enhanced specific activity (Field *et al.*, 2010, Field *et al.*, 2012) production and/ or solubility /diffusion (Rouse *et al.*, 2012). Regardless of the specific underlying basis for the enhanced bioactivity of a particular derivative, any enhanced feature has the potential to be exploited in food or medicine. This is especially true when one considers the number of applications which are available to lantibiotics, and nisin in particular (Cotter *et al.*, 2005).

Preliminary screening of this bank resulted in the identification of 23 derivative producers with enhanced bioactivity. Of these 23, producers of nisin derivatives containing SLS, AAK or NAI within the hinge region were brought forward for further characterisation. In the case of the SLS-containing example, the selection of a strain producing nisin with a serine (S) at positions 20 and 22 was consistent with observations made by Field *et al.* (2008) and Rouse *et al.* (2012), who previously established that the introduction of this hydrophilic amino acid at position 22 and 20 can result in enhanced bioactivity. Field and co-workers have also previously noted that the introduction of a leucine (L) at position 21, in several instances, lead to relatively high levels of activity. With respect to the NAI-containing derivative, nisin A naturally contains an asparagine (N) at position 20, the

benefits of incorporating alanines will be discussed in greater depth below and, although there is no precedent for an enhanced derivative containing an isoleucine (I) at position 22, its introduction at the other two hinge positions has had varying effects on bioactivity (Field *et al.*, 2008). The introduction of alanines in the AAK-containing peptide will be discussed below.

Although this is not the first study which has fully randomised the nisin 'hinge' region, it is the first to do so in the context of the full length nisin peptide. Previously, Plat and co-workers (2011) randomised all three positions in a truncated form of nisin, i.e. nisin-(1-22), and found when they analysed 16 of the active derivatives that the size of the zone was directly, for the most part, proportional to the amount of the prepeptide produced. However, in that instance the derivatives that exhibited the largest zones of activity both contained the aromatic amino acid tryptophan (W); AWR and WRA. In contrast, in this study none of the peptides produced by the 'hinge' mutants that displayed enhanced bioactivity contained aromatic amino acids. The differences between the composition of the hinges in enhanced peptides from these respective studies suggest that the hinge is performing a different role in the truncated, relative to the intact, peptide and that in the latter instance the impact on the hinge residues on the C-terminal domain is critical. Despite these differences, it is apparent that there is a consistent absence of negatively charged amino acids from within the hinge of all peptides and strains exhibiting enhanced bioactivity. This is also consistent with the previous studies of Field *et al.* (2008) and Yuan *et al.* (2004).

Well diffusion assays using purified forms of the SLS-, AAK- and NAI-containing peptides established that enhanced bioactivity was attributable to an enhanced diffusion in agar in a manner similar to that previously reported by Rouse

et al. (2012). This trait was previously noted as being a valuable one in that such a peptide performed better than wild-type nisin A with respect to controlling *Listeria monocytogenes* in a food system. The identity of these, and other, changes that occurred in the hinge region of peptides associated with enhanced bioactivity in the preliminary screening also provided a further insight into the flexibility of the hinge and revealed distinct patterns (Tables 3.3 and 3.4). The frequency with which alanine appears was particularly notable. Among the strains that exhibited enhanced bioactivity, alanine was the amino acid that was most frequently located at positions 20 (30%) and 21 (22%) and was also frequently identified at position 22 (13%). It was also noted that a pair of alanines was located in the hinge region of peptides from three strains exhibiting enhanced bioactivity. The AAK-containing peptide is a perfect example of this pattern. The third residue, lysine, is conserved across all natural variants of nisin; nisin A (Kaletta & Entian, 1989), nisin Z (Mulders *et al.*, 1991), nisin Q (Zendo *et al.*, 2003), nisin F (de Kwaadsteniet, *et al.*, 2008), nisin U and U2 (Wirawan *et al.*, 2006) and most recently nisin P (Zhang *et al.*, 2012). The enhanced bioactivity of the corresponding strain and the specific activity in agar of the corresponding peptide was particularly apparent against *S. aureus* RF122 (Tables 3.6 and 3.7). Despite the conserved nature of this lysine, this study and others (Field *et al.*, 2008, Rouse *et al.*, 2012) have demonstrated that this residue can be changed. On the basis of these observations it was decided to bioengineer a strain to produce a peptide in which the ‘hinge’ would consist wholly of alanines. The fact that these changes enhanced the bioactivity of the associated strain means that this is the first instance upon which a characteristic of nisin has been enhanced through rational design. The additional hinge derivative to result from rational design contained a hinge consisting of SAA. The creation of this peptide was targeted due to the

previous observation that enhanced bioactivity was evident in strains that produced peptides with a serine at position 20 of the nisin peptide, including SMT, SLS and SVA (Rouse *et al.*, 2012). The bioactivity of the resultant strain (Table 3.6) was also greater than that of nisin wild-type producer against all indicators tested. The fact that the newly introduced serine remained unmodified is in agreement with observations made previously by Lubelski (2009), where it was suggested that serines in positions immediately preceding lanthionine bridges remain unmodified. The fact that the addition of small chiral amino acids to the hinge resulted in an increase in bioactivity may be attributable to an increase in ‘hinge’ flexibility. In contrast, the production of the achiral glycine ‘hinge’ may confer a structurally weak hyper flexible ‘hinge’ lacking in any distinguishable conformity through misfolding. It should also be noted that, while we focused on the observation that alanine and serine were frequently identified across all hinge residues in strains with enhanced bioactivity, there may be merit in designing residues whereby the amino acid at each respective location is optimised. Indeed, the positively charged histidine was found very frequently at position 20 in these strains while, at position 21, the hydrophobic residues valine, leucine and isoleucine are very prominent. The identity of the residues located at position 22 was more variable.

Ultimately, the ‘hinge’ region of nisin has again been established to be a worthy target with respect to bioengineering to enhance bioactivity. The benefits of incorporating small chiral amino acids were particularly apparent leading to, for the first time, the rational design of nisin ‘hinge’ derivatives with enhanced properties.

3.6 Acknowledgements

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3.8 Tables and Figures

Table 3.1 Plasmids & strains used in used in this study

Plasmid/Strains	Characteristic	Reference/Source
pDF05	pCI372 with <i>nisA</i>	Field <i>et al.</i> (2008)
pDF05 AAK	pDF05 with N20A/M21A substitution in <i>nisA</i>	This study
pDF05 AAA	pDF05 AAK with K22A substitution in <i>nisA</i> AAK	This study
<i>L. lactis</i> NZ9800	<i>L. lactis</i> NZ9700 Δ <i>nisA</i>	Rea <i>et al.</i> (2011)
<i>L. lactis</i> NZ9800 pDF05	Wild-type nisin A producer	Field <i>et al.</i> (2008)
<i>E. coli</i> Top10	Intermediate cloning host	Invitrogen
<i>M. smegmatis</i> MC2155	Model microorganism for slow-growing mycobacteria species	ATCC
<i>S. agalactiae</i> ATCC 13813	Indicator strain	ATCC
<i>L. lactis</i> ssp <i>cremoris</i> HP	Indicator strain	UCC Culture Collection
<i>S. aureus</i> RF122	Bovine mastitis-causing isolate	Herron-Olson <i>et al.</i> (2007)

ATCC: American Type Culture Collection, UCC: University College Cork

Table 3.2 Oligonucleotides used in this study.

Oligonucleotide	Sequence
NisAXXXHingeFor	5'- PHO TGATGGGTTGT NNKNNKNNK ACAGCAACTTGTCATTGTAGT -3'
NisAXXXHingeRev	5'- CAAGTTGCTGTMNNMNNMNNACAACCCATCAGAGCTCCTGT -3'
pCI372Rev	5'- ACCTCTCGGTTATGAGTTAG -3'
For Primer (AAA)	5'- GTTGTGCTGCG GCA ACAGCAACTTGTCATTGTAGTATTAC -3'
Rev Primer (AAA)	5'- CAAGTTGCTGTT TGCC CGCAGCACAACCCATCAGAGCTCCTGT -3'
AAA Check Primer For	5'- CTGATGGCTTGTGCTGCG GCA -3'
SAA HC For	5'- TGATGGGTTGT TCA GCGGCTACAGCAACTTGTCATTGTAGT -3'
SAA HC Rev	5'- GCTGTAGCCGCT TGA ACAACCCATCAGAGCTCCTGTTTACA -3'
SAA Forward SLT Codon	5'- TGATGGGTTGT TCG GCGGCTACAGCAACTTGTCATTGTAGT -3'
SAA Reverse SLT Codon	5'- GCTGTAGCCGCC GCA ACAACCCATCAGAGCTCCTGTTTACA -3'
GGG Forward Primer	5' GATGGGTTGT GGAGGTGGA ACAGCAACTTGTCATTGTAGTA '3
GGG Reverse Primer	5' AGTTGCTGTT TCCACCTCC ACAACCCATCAGAGCTCCTGTTT '3
GGG Check Primer	5' TCTGATGGGTTGT GGAGGTGGA '3

PHO – 5'- Phosphate modification. Emboldened – Degenerate codons, emboldened & underlined – locations for site-directed mutagenesis

Table 3.3 Nisin A ‘hinge’ variants with enhanced bioactivity identified through the initial screen.

Variant	Molecular Mass	Variant	Molecular Mass
HVS	3304	AIT	3266
MAQ	3311	QVQ	3336
ASS	3226	SMT	3300
PVN	3291	HSQ	3332
ASV	3238	HAA	3260
HLA	3301	SIN	3294
NAI	3279	PQK	3334
ANP	3263	AQV	3278
SLS	3268	HSQ	3332
AAI	3236	PNA	3262
AAK	3250	NQV	3321
		HLS	3308

The three letter code corresponds to the amino acids located at each of the three ‘hinge’ sites.

Table 3.4 Frequency with which amino acids are located at each hinge site among the derivatives presented in Table 3.3.

Position 20 (% frequency)	Position 21 (% frequency)	Position 22 (% frequency)
Alanine 7 (30.4)	Alanine 5 (21.7)	Serine 4 (17.4)
Histidine 6 (26.1)	Serine 4 (17.4)	Glutamine 4 (17.4)
Proline 3 (13.0)	Valine 3 (13.0)	Alanine 3 (13.0)
Serine 3 (13.0)	Leucine 3 (13.0)	Valine 3 (13.0)
Asparagine 2 (8.7)	Glutamine 3 (13.0)	Isoleucine 2 (8.7)
Glutamine 1 (4.3)	Asparagine 2 (8.7)	Asparagine 2 (8.7)
Methionine 1 (4.3)	Isoleucine 2 (8.7)	Lysine 2 (8.7)
	Methionine 1 (4.3)	Threonine 2 (8.7)
		Proline 1 (4.3)

Table 3.5 Actual and expected frequencies of hinge amino acids from randomly selected representatives of the hinge mutant bank

Amino Acid Residue	Actual Frequency (%)	Expected Frequency (%)
Serine	8.33	9.38
Alanine	6.67	6.25
Asparagine	6.67	3.13
Lysine	6.67	3.13
Tyrosine	6.67	3.13
Arginine	5.00	9.38
Glutamine	5.00	3.13
Glycine	5.00	6.25
Histidine	5.00	3.13
Isoleucine	5.00	3.13
Methionine	5.00	3.13
Stop	5.00	3.13
Valine	5.00	6.25
Aspartic Acid	3.33	3.13
Cytosine	3.33	3.13
Glutamic Acid	3.33	3.13
Leucine	3.33	9.38
Proline	3.33	6.25
Threonine	3.33	6.25
Tryptophan	3.33	3.13
Phenylalanine	1.67	3.13

Table 3.6 Deferred Antagonism Results

		Deferred Antagonism		
		Zone Area (mm ²)	p- value	Relative to WT (%)
<i>L. lactis</i> HP	WT	299.58 ± 7.04		
	AAA	492.83 ± 40.27	0.012	164.51
	AAK	408.89 ± 18.72	0.005	136.49
	SAA	483.14 ± 33.89	0.009	161.28
	SLS	370.75 ± 28.28	0.042	123.76
	NAI	439.52 ± 47.88	0.034	146.71
<i>S. aureus</i> RF122	WT	138.31 ± 3.36		
	AAA	292.24 ± 11.46	0.001	211.29
	AAK	292.44 ± 10.36	0.001	211.43
	SAA	236.28 ± 13.53	0.004	170.83
	SLS	153.93 ± 7.15	0.045	111.29
	NAI	16.3.23 ± 10.95	0.049	118.02
<i>S. agalactiae</i> ATCC 13813	WT	226.55 ± 2.96		
	AAA	401.32 ± 15.62	0.002	177.14
	AAK	232.00 ± 21.58	0.706	102.41
	SAA	407.98 ± 18.452	0.003	180.08
	SLS	209.45 ± 17.11	0.223	92.45
	NAI	318.81 ± 7.915	0.034	140.72
<i>M. smegmatis</i> MC2155	WT	51.31 ± 10.08		
	AAA	277.65 ± 60.42	0.02	541.09
	AAK	199.47 ± 32.36	0.009	388.73
	SAA	303.85 ± 59.93	0.016	592.15
	SLS	230.31 ± 20.31	0.001	448.84
	NAI	270.04 ± 58.30	0.02	526.26

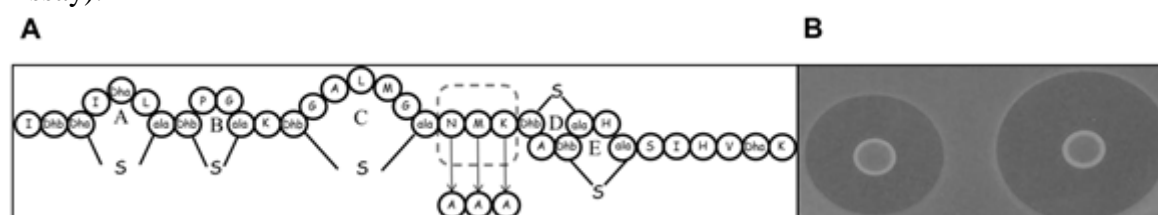
The zone of inhibition is expressed as the area of the zone of inhibition minus the area of the ‘spot’ in mm².

Table 3.7 Specific Activity Results

		100 mg L ⁻¹ Agar Diffusion			MIC
		Zone Area (mm ²)	p-value	Relative to WT (%)	Specific Activity as % of WT
<i>L. lactis</i> HP	WT	267.69 ± 9.21			
	AAA	416.00 ± 17.99	0.001	155.41	100
	AAK	320.08 ± 14.20	0.009	119.57	50
	SAA	344.76 ± 18.50	0.008	138.71	50
	SLS	321.93 ± 22.43	0.038	120.26	25
	NAI	371.32 ± 19.60	0.004	128.79	100
<i>S. aureus</i> RF122	WT	99.34 ± 12.33			
	AAA	155.77 ± 3.30	0.011	156.81	25
	AAK	178.23 ± 13.39	0.002	179.42	50
	SAA	130.60 ± 4.46	0.036	131.47	25
	SLS	98.82 ± 10.14	0.957	99.47	<6
	NAI	129.75 ± 11.36	0.035	130.61	25
<i>S. agalactiae</i> ATCC 13813	WT	246.31 ± 2.74			
	AAA	305.58 ± 2.39	0.000	124.06	200
	AAK	234.55 ± 4.23	0.021	95.23	50
	SAA	301.62 ± 8.18	0.004	122.46	100
	SLS	226.05 ± 4.10	0.003	91.78	25
	NAI	291.52 ± 8.46	0.007	118.36	200
<i>M. smegmatis</i> MC2155	WT	67.79 ± 4.308			
	AAA	149.24 ± 4.776	0.000	220.15	ND
	AAK	105.73 ± 6.930	0.003	155.96	ND
	SAA	101.19 ± 11.510	0.026	149.27	ND
	SLS	119.56 ± 8.59	0.003	176.36	ND
	NAI	78.50 ± 8.16	0.137	115.8	ND

MIC: Minimum Inhibitory Concentration ND: Not Determined

Figure 3.1 A - Nisin A mature peptide, B - AAA Producer (Deferred Antagonism Assay).



Panel A. Dha – Dehydroalanine; Dhb – Dehydrobutyrine. Hinge region highlighted. Five (β-methyl)lanthionine rings labelled A-E. Arrows denote amino acid changes to produce AAA derivative in the nisin hinge region. Panel B. Zones of inhibition produced by *L. lactis* NZ9800 pDF05(left) and AAA 'hinge' variant (right) against *L. lactis* HP.

Chapter IV

Bioengineering to create a trypsin resistant Nisin A derivative

Brian Healy, Des Field, Paula M O'Connor Colin Hill, Paul D. Cotter and R. Paul Ross

Manuscript in Preparation

4.1 Abstract

Nisin A is a 34 amino acid Class 1 lantibiotic. It has a long and successful history of use in the food industry having been approved as a food preservative by the EU, the WHO and the USFDA. Lantibiotics also have potential as alternatives to clinical antibiotics due to their effectiveness against many human, including multi-drug resistant pathogens. However, their somewhat proteolytically labile nature results in their breakdown during transit in the gastro-intestinal tract, a major hurdle in nisin's potential use in a clinical setting.

Numerous studies have demonstrated the amenability of the prototypic lantibiotic nisin to genetic engineering in order to improve on its already impressive functionality and antimicrobial activity. In this study we employed both site-specific and site-saturation PCR based mutagenesis techniques to confer onto the peptide a greater ability to resist trypsin digestion, through the substitution of trypsin sensitive lysine and arginine residues. By targeting the lysine 12 amino acid of a nisin A derivative in which the innate hinge residues have been replaced by a triplet of alanines (AAA), a derivative containing a lysine to methionine substitution at this position, K12M-AAA, exhibited both enhanced bioactivity and a loss of trypsin sensitivity.

4.2 Introduction

The lantibiotic (**lantionine-containing antibiotic**) class of bacteriocins (Rea *et al.*, 2011) are a distinctive class of cationic antimicrobial peptides due to the presence of unusual amino acids found within their structures arising from a number of post-translational modifications. The most thoroughly studied lantibiotic, the *Lactococcus lactis* produced nisin A, contains five thio-ether bridged amino acids; one lantionine bridge (Lan) and four β -methyllanthionine bridges (meLan) (Sahl *et al.*, 1995). These bridges result from the coupling of dehydrated serines (dehydroalanines) and threonines (dehydrobutyrines) with proximal cysteines through the combined action of a dehydratase (NisB) and a cyclase (NisC) and provide the mature/ modified peptides with increased protection from thermal and proteolytic stress (Suda *et al.*, 2010).

The importance of lantionine bridges in protecting lantibiotics from proteases has been demonstrated through the study of peptides from which lantionine bridges have been removed (Bierbaum *et al.*, 1996) (Suda *et al.*, 2010). This removal lead to destabilization of the peptides, exposing the otherwise sheltered cleavage sites to proteases. Notably, while lantibiotics exhibit an enhanced resistance to proteolytic degradation relative to unmodified bacteriocins, they are nonetheless ribosomally synthesised peptides and thus remain more sensitive to proteases than non-ribosomal antibiotics (Caboche *et al.*, 2008). In the context of the use of lantibiotics in the food industry as a preservative and shelf-life extender (Deegan *et al.*, 2006), this proteolytic sensitivity, and in particular sensitivity to trypsin and chymotrypsin in the small intestine, has been viewed as advantageous as it ensures that the peptides are broken down before they negatively influence the gut microbiota (O'Shea *et al.*, 2010). However, this perceived advantage can be viewed

as a disadvantage in the context of the use of nisin, and other lantibiotics, as alternatives to antibiotics to control clinical or veterinary gastrointestinal tract (GIT) pathogens (Piper *et al.*, 2009, Jabés *et al.*, 2011, Piper *et al.*, 2012, Cotter *et al.*, 2013) such as colonic *Clostridium difficile* or colonization by vancomycin-resistant enterococci (VRE). In order to circumvent the proteolytic sensitivity of bacteriocins, encapsulation has been investigated as a means of facilitating transit through the GIT. Indeed *in vitro* studies have found that a pectin/ HPMC (Hydroxypropyl methyl cellulose) envelope is suitable for delivery of nisin to the colon (Ugurlu *et al.*, 2007). It should also be noted that not all bacteriocins are trypsin sensitive. One such exception is salivaricin D, produced by *Streptococcus salivarius* 5M6c, which is intrinsically resistant to trypsin due to the absence of lysine and arginine residues within its sequence (Birri *et al.*, 2012).

Another consequence of the ribosomal, i.e. gene encoded, nature of lantibiotics is that they can be relatively easily altered through gene manipulation. Indeed, an ever growing number of studies have shown how this has been exploited specifically for nisin to improve various physicochemical traits and specific activity against a number of very relevant Gram-positive and negative pathogenic bacteria including MRSA and *Clostridium difficile* (Field *et al.*, 2008, Field *et al.*, 2010, Field *et al.*, 2012, Rouse *et al.*, 2012, Healy *et al.*, 2013, Molloy *et al.*, 2013). Here we apply this tolerance of nisin to change in order to bioengineer a derivative that is resistant to trypsin.

More specifically, a dual approach of site-specific and site-saturation mutagenesis technique to introduce amino acid substitutions is employed with the specific aim to create/ unearth active nisin derivatives resistant to trypsin while, importantly, also maintaining antimicrobial activity. With respect to trypsin, nisin

contains three cut sites; lysine at amino acid position 12, asparagine 20 and lysine 22 while asparagine 20, methionine 22 and histidine 31 are chymotrypsin sensitive cut sites (Figure 4.1)(Slootweg *et al.*, 2013). This approach led to the creation of four putatively trypsin or chymotrypsin resistant derivatives of which one, K12M-AAA, (lysine at position 12 replaced by alanine and the three ‘hinge’ associated residues, i.e. N20, M21 and K22, replaced by alanines) was characterised in greater depth and confirmed to be trypsin resistant and to retain antimicrobial activity. To our knowledge this represents the first occasion whereby a rationally designed, bioengineering strategy resulted in the creation of a bioactive trypsin resistant nisin variant.

4.3 Materials and Methods

4.3.1 Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 4.1. *Lactococcus lactis* cultures were grown in M17 agar and broth (both Oxoid) supplemented with 0.5% glucose at 30°C overnight. *Escherichia coli* strains were grown in 1% NaCl (Sigma-Aldrich), 1% Yeast Extract (Merck) and 0.5% Tryptone (Merck) at 37°C overnight with constant shaking. *Streptococcus agalactiae* ATCC 13813 was grown in Tryptic Soy Broth (TSB) (Merck) supplemented with 0.6% (w/v) Yeast Extract (Merck) at 37°C overnight. Where applicable, chloramphenicol (Sigma-Aldrich) was added at 10 µg/ml.

4.3.2 Site-saturation mutagenesis of the K12A

The targeted change of the lysine at position 12 was achieved through a PCR based method using the oligonucleotides NisK12A-AAFor and NisK12ARev (Table 4.2). The plasmid pDF05-AAA (Healy *et al.*, 2013) (Table 4.1), which had been extracted from *dam*⁺ *E. coli* Top10 (Invitrogen) to ensure its methylation, was used as the template for this reaction. Amplification was achieved through a 50 µl PCR reaction consisting of 0.02 U/µl KOD Hot-Start Polymerase (Novagen), 1.5 mM MgSO₄, 0.2 mM of each dNTP, 0.3 µM of both oligonucleotides and approximately 10 ng of template. Cycling conditions were as follows: 98°C for 30 sec, 60.0°C for 15 sec and 72°C for 3.5 min for 40 cycles followed by final extension for 10 min. The resulting product was *DpnI* treated (Stratagene) at 37°C for 3 hours with subsequent cleaning using GeneJet PCR Purification Kit (ThermoScientific). 2.5 µl of mutated pDF05-AAA added to a vial of Top 10 Chemically Competent *E.coli* (Invitrogen). Transformants were selected for on LB Cm¹⁰ agar. pDF05-K12A-AAA was

extracted using a High Pure Plasmid Isolation Kit (Roche), transformed into electro-competent *L. lactis* ssp. *lactis* NZ9800 Δ *nisA* and selected for on GM17 Cm¹⁰ agar. The changes to the *nisA* genes within the corresponding pDF05 derivative were confirmed through DNA sequencing (MWG, Biotech, Germany).

4.3.3 Site-saturation mutagenesis of K12,H31 and combination of both

Site-saturation mutagenesis was achieved through a PCR based method using the oligonucleotide sets NisH31DegFor and NisH31DegRev, and NisK12DegAAFor and NisK12DegREV (Molloy *et al.*, 2013) (Table 4.2) with pDF05 pDF05-AAA extracted from dam⁺ *E. coli* Top10 used as the template on both occasions. Intermediate *E. coli* cloning with subsequent expression in *L. lactis* NZ9800 Δ *nisA* was carried out as outlined in the previous section. Approximately 150 randomly chosen transformants from the respective PCR reactions were assayed through deferred antagonism in order to select for derivatives displaying activity comparable or greater than that of the wild-type nisin A producer.

In order to produce a bank in which both the K12 and H31 positions are randomised on a single derivative, pDF05-K12X-AAA was used as a template for a subsequent H31X saturation. A bank of 2400 *L. lactis* derivatives were picked using Genetix QPIX II-XT colony-picking robot and added to GM17 Cm¹⁰ within 96-well plates, incubated overnight at 30°C and stored at -80°C.

4.3.4 Derivative Identification

Colony mass spectrometric analysis was carried using an Axima TOF² MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) and analyzed in positive-ion linear mode as previously described (Field *et al.*, 2008) to confirm production

and the size of the resulting derivative. DNA sequencing of the *nisA* gene within the pDF05 AAA derivative was carried out (Source Biosciences, Dublin, Ireland) to confirm the desired change had occurred.

4.3.5 Peptide Purification

Two 10 ml overnights of the culture of interest were added to 2 l of Tryptone Yeast (TY) and incubated at 30°C for at least 20 hours. The sample was next centrifuged for 20 min at 8630 g. The cell free supernatant (CFS) was passed through 60g of pre-equilibrated Amberlite XAD16 beads (Sigma-Aldrich) with subsequent washing of the column with 500ml of 30% ethanol and elution in 500ml of 70% isopropanol (IPA) (Fisher) with 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich). Concurrently, the cell pellets was resuspended in 500 ml 70% IPA 0.1% TFA and stirred at room temperature for a minimum of 3 hours followed by centrifugation. This supernatant was combined with the CFS eluate (800 ml) and reduced to a final volume of 250 ml through rotary evaporation (Buchi, Switzerland) and the pH adjusted to 4.5. The sample was further purified and concentrated to 60 ml through a Phenomenex SPE C-18 column. 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 μ m). Peaks were evident within a gradient of 30–50% acetonitrile (Fisher) containing 0.1% TFA. These fractions were pooled and the acetonitrile removed through rotary evaporation and freeze-dried (LABCONCO). To ensure the integrity before subsequent analysis, the peptide purity was determined by MALDI TOF mass spectrometric analysis.

4.3.6 Bioactivity and specific activity assays

Initial screening of the various derivative banks was carried out using the deferred antagonism assay whereby approximately 3µl volumes of the putative producers were ‘spotted’ onto GM17 agar with 10 ng/ml nisin (so as to allow for the possibility that induction may have being affected by bioengineering) and allowed to grow overnight followed by UV treatment. The indicator was seeded at 0.5% (v/v) into the specific growth media with an agar concentration of 0.75% (w/v), poured over the test plate and incubated overnight. Those displaying zones of inhibition comparable to the wild-type producer were identified and purified if the amino acid changes that occurred led to substitution of an enzyme susceptible residue.

Agar well diffusion assays (WDA) were carried out through the addition of 50 µl volumes of 30 µM purified peptide solutions into wells bored into the specific growth media for the indicator in question with a final volume of 50ml and an agar concentration of 0.75%. The plates for kept at 4°C for 3 hours followed by incubation overnight.

The minimum inhibitory concentration assays (MIC) were carried out as stated previously (Healy *et al.*, 2013). In brief, the assay was performed in triplicate in 96-well plates (Sarstedt) pre-treated with bovine serum albumin. 500 nm concentrations of both the wild-type and derivative purified powders were added to the first well and serially diluted in 2-fold increments down to a concentration of <1 nmol. An overnight culture of the indicator was subcultured and incubated until reaching an OD 600 nm of 0.5 and diluted to 10⁵ cfu/ml in 200 µl, which was added to each test well. The plates were incubated for 16 hours and the MIC read as the lowest peptide concentration where growth was not visible.

4.3.7 Trypsin Digestions

20 µg of New England Biolabs Trypsin Ultra, Mass Spectrometry Grade (P81015) was resuspended into 20 µl of 25 mM ammonium bicarbonate pH 8.0 to give a stock solution of 1 mg/ml. RP-HPLC purified nisin A and Nisin K12M-AAA peptides were resuspended at 1 mg/ml in Milli Q water. The peptide purity was checked using MALDI TOF mass spectrometry (MS).

Trypsin digests were set up as follows. 50 µl of 1 mg/ml peptide and 50 µl of 200 µg/ml trypsin were added to 100 µl of ammonium bicarbonate buffer. Control digests were set-up by substituting the peptides for ammonium bicarbonate buffer. Digests were incubated on a heating block at 37°C for 48 hrs. MALDI-TOF MS was carried out at T0, T24 and T48 hrs with the addition of fresh 1 mg/ml trypsin (10 µl final volume) added at T29. 4 µl aliquots, diluted to 50 µl, were taken for well diffusion assays (WDA) using *L. lactis* ssp. *cremoris* HP as an indicator.

135 µl of both digestions was taken at T0 and T48 and RP-HPLC analysis of the each was run on a Dionex micro analytical C18 RE-HPLC column (2.1 x 150 mm, 100Å, 3 µm) running on a 10-45% acetonitrile 0.1% TFA gradient over 40 minutes.

4.4 Results

A nisin derivative peptide that contained the alanine(A)-alanine(A)-alanine(A) hinge (Positions 20, 21 and 22) with a lysine to alanine change at position 12 was designed with a view to making a trypsin resistant form of the peptide. A derivative containing a AAA ‘hinge’ is advantageous on two levels; a peptide containing these substitutions has previously been shown to exhibit increased bioactivity and specific activity relative to nisin A (Healy *et al.*, 2013) while also, it lacks two of the three trypsin cleavage sites. The remaining cleavage site is the lysine at position 12, which has also been shown to be amenable to change as evidenced by the enhanced (bio)activity of a K12A-containing derivative (Molloy *et al.*, 2013). To generate a putatively trypsin-resistant derivative, these changes were combined in a peptide designated K12A-AAA using the oligonucleotides NisK12A-AAFor and NisK12ARev (Table 4.2). To determine if other changes at the K12 site could yield additional peptides of interest, a site saturation reaction was completed using the primers NisK12DegAAFor and NisK12DegREV (Table 4.2). From this, K12M-AAA emerged as an attractive candidate for more in depth characterisation due to the zone of inhibition it produced in a deferred antagonism assay (Table 4.3). Nisin derivatives containing an ‘AAA’ hinge and a K12 mutation retain a single chymotrypsin cut-site, the histidine residue at position 31. To expand on the number of protease resistant derivatives at our disposal, a double site saturation mutagenesis was undertaken by using the previously mutated pDF05K12X from the *E. coli* Top 10 plasmid pool as a template for a reaction using the NisH31Deg For and NisH31Deg Rev primer set in order to produce a bank of derivatives in which both K12 and H31 sites are randomised. Both sites were randomised simultaneously in order to increase the chances of generating a trypsin/ chymotrypsin derivative that

retains activity. As the total number of unique codon combinations of a two amino acid, NNK degenerative primer set produced bank is 1024 (32 x 32) it was decided to screen a bank of 2400 putative producers in order to obtain 2X coverage. Two derivatives that retained bioactivity (Table 4.3) and which were predicted to be chymotrypsin resistant emerged from this screen, i.e. K12A-AAA-H31R and AAA-H31R. However, the H31R substitution present in both peptides results in the re-introduction of a trypsin cleavage site. As none of the four lead derivatives generated to this point, K12A-AAA, K12M-AAA, K12A-AAA-H31R or AAA-H31R, were predicted to be resistant to both proteases, it was decided to create another derivative in which a proline was inserted directly after the R31 residue, so as to negate the trypsin sensitivity (Keil's Rule) (Keil, 1992), yielding K12A-AAA-H31R-V32P.

The bioactivity of each derivative was determined in triplicate using both *L. lactis* HP and *S. agalactiae* ATCC 13813 as the indicators. The zone size was expressed as a percentage relative to that produced by the wild-type (Table 4.3). K12A-AAA gave a zone size of 107% (p-value 0.119) and 104% (p-value 0.681) against *L. lactis* HP and *S. agalactiae* ATCC 13813, respectively, while K12A-AAA-H31R generated zones 77% (0.059) and 86% (0.165) that of wild-type against the same indicators. Both K12M-AAA and AAA-H31R displayed bioactivity at levels statistically greater than wild-type. The former giving zone sizes of 125% (p-value 0.019) and 116% (0.041) against *L. lactis* HP and *S. agalactiae* ATCC 13813, respectively, while the latter displayed bioactivity that was 126% (0.027) and 135% (0.003), respectively, of that of the wild-type against the same targets. K12A-AAA-H31R-V32P did not give a zone of inhibition. Based on these results both K12M-AAA and AAA-H31R were brought forward for purification and further assessment of their activity.

The activity of the purified peptides was first assessed using equimolar concentrations of the peptides (30 μ M) and agar well diffusion assays. AAA-H31R and K12M-AAA produced zones against *L. lactis* HP that were not significantly different from that produced by the wild-type peptide (98%, p-value 0.298 and 99%, p-value 0.568, respectively) Table 4.4. When using *S. agalactiae* ATCC 13813 as the indicator, AAA-H31R produced a zone that was 120% (p-value 0.001) that of the wild-type while K12M-AAA produced a zone with an 80% (p-value 0.001) relative size. As these assays reflect both the activity of the peptides and their ability to diffuse through a complex media such as agar, further assessments focused on specific activity only. These broth-based minimum inhibitory concentration (MIC) assays were again carried out using the HP and ATCC 13813 strains (Table 4.4). K12M-AAA had an MIC of 31.3 nM, compared to 15.6 nM for wild-type nisin A, against HP. The MIC of AAA-H31R against the same strain was 62.5 nM. When *S. agalactiae* ATCC 13813 was used as an indicator, the reduction in the activity of AAA-H31R (125 nM) relative to wild-type (62.5 nM) was less. K12M-AAA had an MIC of 95.5 nM against the same strain.

As a consequence of its greater specific activity and our particular interest in the creation of trypsin resistant nisinA derivatives, purified K12M-AAA was selected to be brought forward for trypsin digestion assays. This was achieved through assays whereby both the derivative peptide and the wild-type were digested for 48 hours at 37°C at a ratio of 5:1 peptide to trypsin with samples taken for WDAs using *L. lactis* HP as an indicator at T0, T24 and T48 hrs. Concurrently, digests were set-up whereby trypsin was replaced by ammonium bicarbonate buffer as a control (in order to view possible non-trypsin associated breakdown of the peptide during the run-time of the assay). From Figure 4.2 A, the detrimental impact of trypsin on

the nisin A peptide is evident as peptide that was pre-digested for 48 hrs did not produce a zone of inhibition. This contrasts with the ammonium bicarbonate control where only a slight decrease in the zone size was observed. When K12M-AAA was tested under identical conditions a substantial level of inhibitory activity was retained, even after 48 hours in the presence of trypsin (Figure 4.2 B). The presence of a zone at T48 for K12M-AAA confirms its trypsin resistance.

In order to more fully investigate this phenomenon, RP-HPLC was carried out to purify nisin A and K12M-AAA peptides before and after exposure to trypsin. Figure 4.3 displays the chromatographs corresponding to nisin A before (T0; top) and after (T48; bottom) treatment with trypsin. Most noticeable from both chromatographs is the dramatic decrease in the size of the peak corresponding to intact nisin A between T0 (A) and T48 (B) which corresponds to the decrease in the zone of inhibition in Figure 4.2 A. Also visible in Figure 4.3 are the various fragments resulting from the trypsin digestion, the most prominent of which are nisin 1-12 (1150 Da) and nisin 21-34 (1492 Da). Figure 4.4 displays the corresponding K12M-AAA chromatographs i.e. before (T0; A) and after (T48; B) exposure to trypsin. From these chromatographs the trypsin resistance of K12M-AAA is clearly apparent.

4.5 Discussion

There have been some previous attempts to decrease the trypsin-sensitivity of bacteriocins through amino acid substitution. This can be achieved by either substituting arginine, lysine or adjacent residues with non-sensitive residues or by decreasing the conformational flexibility of the peptide therefore limiting exposure to the proteases catalytic cleft both of which can negatively influence the bioactivity of bacteriocins. For example, proteolytic resistance has been increased in derivatives of the lantibiotic gallidermin using the former approach but this was accompanied with a dramatic decrease in antimicrobial activity (Ottenwälder *et al.*, 1995). One such gallidermin derivative was Dhb14P(threonine at position 14 substituted by proline) which follows a lysine residue at position 13. This amino acid orientation negates the action of trypsin (Keil, 1992). The former approach has also been successfully implemented in the synthesis of trypsin-resistant derivatives of one of the peptides of the two component bacteriocin Class IIb bacteriocin salivarcin P (O'Shea *et al.*, 2010), leading to a histidine-substituted derivative that exhibited only a modest decrease in activity. While the lantibiotic group of bacteriocins are an attractive candidate to fill the void in novel antibiotic discovery against the rise of multi-drug resistant pathogens, their sensitivity to a number of proteases can be viewed as a shortfall. Although a partial intrinsic protease protection is afforded by the eponymous lanthionine bridges, nisin A remains trypsin sensitive, as is clearly apparent (Figure 4.2 A). When digested with trypsin it is observed that the peak corresponding to the intact lantibiotic (Figure 4.3) is reduced drastically after 48 hours with the emergence of a number fragments corresponding to those predicted to arise as a consequence of trypsin digestion. Notably the identification of a peak corresponding to nisin A 21-34 (Peak E 1491 Da, Figure 4.3) confirms that the

asparagine residue at position 20 is trypsin sensitive, as previously reported by Slootweg *et al* (2013).

In this study, by exploiting the gene encoded nature of bacteriocins and through applying the knowledge base gained through previous nisin A mutagenesis studies (Healy *et al.*, 2013, Molloy *et al.*, 2013), a number of nisin derivatives were engineered which were enhanced in comparison with nisin A in terms of bioactivity while conferring onto it trypsin or chymotrypsin resistance and therefore improved functionality. This resistance would facilitate transit through the small intestine in which (chymo)trypsin is found and allow such derivatives to arrive intact in the large intestine tract to control undesirable gut microorganisms, such as *C. difficile* (which is very sensitive to nisin (Field *et al.*, 2010)). The fact that the derivatives continue to be ribosomal peptides, and are likely to be eventually degraded by other proteases present in the large intestine (Gibson *et al.*, 1989), including those produced by the gut microbiota, is significant in light of the importance of the gut microbiota to human health and the complexity of the bacterial community relationships within has gained huge attention (Walsh *et al.*, 2014).

Interestingly, both regions of the nisin A peptide that are sensitive to trypsin cleavage, i.e. K12 and the asparagine and lysine of the 'hinge' region, and which are the targeted for modification in this study are thought to be involved in the conformational flexibility of the peptide and subsequent antimicrobial activity (Breukink *et al.*, 1997, Wiedemann *et al.*, 2001, Molloy *et al.*, 2013). This flexibility is important as the bactericidal mode of action of nisin is twofold with the N-terminal region involved in binding to a precursor of peptidoglycan synthesis leading to a conformational change facilitated by the 'hinge' region which results in pore formation by the C-terminal end. Thus, manipulations to increase trypsin resistance

had the potential to reduce flexibility and, in turn, antimicrobial activity and so it was important to specifically screen peptides in which target sites for proteases were removed but which retained significant levels of activity. Bioactivity at levels comparable to that of nisin A was achieved through the bioengineering of a derivative whereby a hinge consisting of three alanines was combined with a lysine to alanine substitution at position 12 leading to the formation of K12A-AAA. Through a site saturation technique it was found that the incorporation of the hydrophobic residue methionine at this position, K12M-AAA, lead to statistically significant increase in bioactivity compared to nisin A. Also from this study, two possible candidates have emerged from the site saturation mutagenesis approach on histidine 31, K12A-AAA-H31R and AAA-H31R which theoretically should show a lack of sensitivity to chymotrypsin. Although outside the scope of this study, the enhanced bioactivity which has been attributed to AAA-H31R clearly merits further study in the future.

In all cases, it is notable that there is a decrease in the overall charge of nisin by two without having a detrimental effect on the bioactivity. This is despite studies suggesting that the overall positive charge of nisin aids in the binding of the peptide to the negatively charged phospholipid layer of sensitive cells (van Kraaij *et al.*, 1999). More specifically, it was claimed that the +4 charge of the C-terminus region plays an important role in this respect. However, as can be seen with the bioactive derivatives generated in this study, a decrease in charge to +3 in this region also does not lead to deleterious consequences.

The well assays and RP-HPLC chromatographs from the trypsin digestion, or lack of digestion, of K12M-AAA (Figure 4.2 B and Figure 4.4) demonstrates how the functionality of nisin can be improved upon by bioengineering. From the well

diffusion assays and chromatographs it is clearly evident that the derivative peptide has retained activity/ integrity, while the wild-type has not and it is clear that the goal of the study has been achieved.

In conclusion, in this study we improved upon two already enhanced nisin derivatives, AAA and K12M, through their combination and have shown that the resulting derivative, K12M-AAA, maintains considerable antimicrobial activity while gaining a resistance to trypsin. This could be seen as a major step forward for the use of lantibiotics as systemic anti-bacterials. From a more fundamental lantibiotic viewpoint these derivatives may provide the blueprint for the design of narrow spectrum nisin derivatives, which transit the small intestine but are even less likely to impact on the gut microbiota, through further bioengineering studies.

4.6 Acknowledgements

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4.8 Tables and Figures

Table 4.1 Plasmids & strains used in this study

Plasmid/Strains	Characteristic	Reference/Source
pDF05	pCI372 with <i>nisA</i>	Field <i>et al.</i> (2008)
pDF05-AAA	pDF05 with hinge NMK to AAA substitution	Healy <i>et al.</i> (2013)
pDF05-K12A-AAA	pDF05-AAA with K to A substitution at position 12	This study
pDF05-K12X-AAA	pDF05-AAA randomly saturated at position 12	This study
pDF05-AAA-H31X	pDF05-AAA randomly saturated at position 31	This study
<i>L. lactis</i> NZ9800	<i>L. lactis</i> NZ9700 Δ <i>nisA</i>	Kuipers <i>et al.</i> (1993)
<i>L. lactis</i> NZ9800 pDF05	Wild-type nisin A producer	Field <i>et al.</i> (2008)
<i>E. coli</i> Top10	Intermediate cloning host	Invitrogen
<i>S. agalactiae</i> ATCC 13813	Indicator strain	ATCC
<i>L. lactis</i> ssp. <i>cremoris</i> HP	Indicator strain	UCC Culture Collection

ATCC: American Type Culture Collection, UCC: University College Cork

Table 4.2 Oligonucleotides used in this study.

Oligonucleotide	Sequence
NisK12A-AA For	5'-CCCGGTTGTGCTACAGGAGCTCTGATGGGTTGTGCTGC -3'
NisK12ARev	5'-AGCTCCTGTAGCACAAACCGGGTGTACATAGCGAAATACT-3'
NisH31DegFor	5'-PHO-TGTAGTATTNNKGTAAGCAAATAATCTAGAGTCGACCT-3'
NisH31DegRev	5'-TTTGCTTACMNNAATACTACAATGACAAGTTGCTGT-3'
NisK12DegAAFor	5'-CCCGGTTGTNNKACAGGAGCTCTGATGGGTTGTGCTGCG-3'
NisK12DegREV	5'-AGCTCCTGTMNNACAACCGGGTGTACATAGCGAAATACT -3'
NisH31RV32PFor	5'-TGTAGTATTCGGCCCAGCAAATAATCTAGAGTCGACCTG-3'
NisH21RV32PRev	5'-TTTGCTGGGCCGAATACTACAATGACAAGTTGCTGT-3'

PHO – 5' - Phosphate modification.

Table 4.3 Deferred Antagonism Results

Indicator	Producer	Deferred Antagonism		
		Zone Area (mm ²)	Zone size relative to WT (%)	p- value
<i>L. lactis</i> HP	WT	311.96		
	K12M-AAA	390.22	125.08	0.019
	AAA-H31R	393.87	126.26	0.027
	K12A-AAA	332.41	106.56	0.119
	K12A-AAA-H31R	239.64	76.82	0.059
	K12A-AAA-H31R-V32P	No Zone		
<i>S. agalactiae</i> ATCC 13813	WT	234.04		
	K12M-AAA	270.97	115.78	0.041
	AAA-H31R	315.63	134.86	0.003
	K12A-AAA	244.20	104.34	0.681
	K12A-AAA-H31R	201.09	85.92	0.165

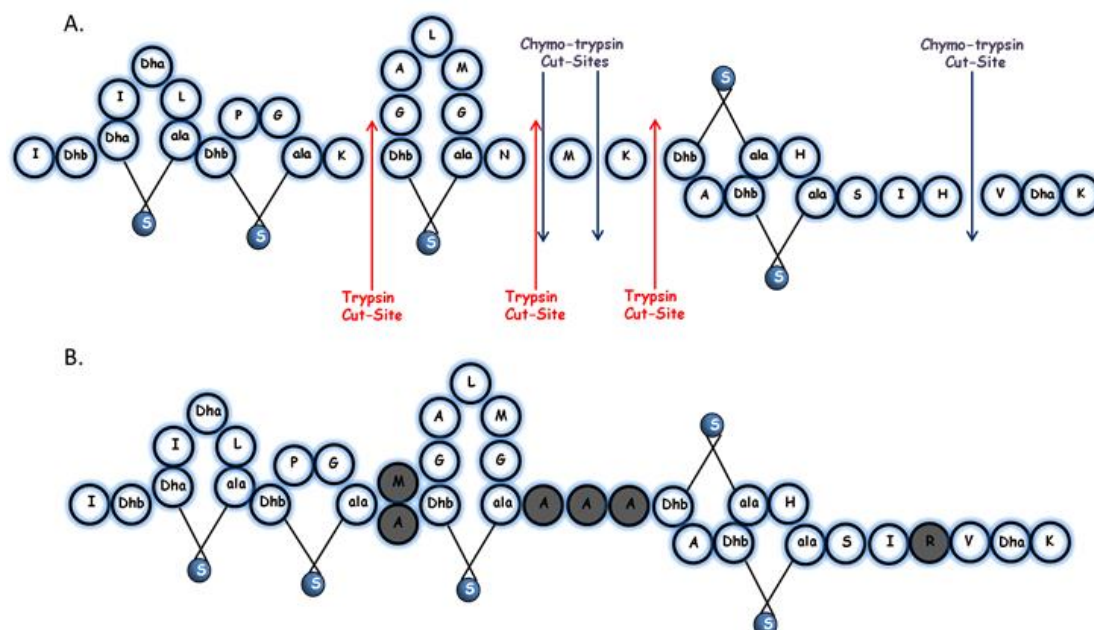
The zone of inhibition is expressed as the area of the zone of inhibition minus the area of the 'spot' in mm²

Table 4.4 Specific Activity Results

Indicator	Producer	30µM Agar Diffusion			MIC µg/ml (nM)
		Zone Area (mm ²)	p-value	Relative to WT (%)	
<i>L. lactis</i> HP	WT	272 ± 4.86			3.14 (15.6)
	AAA-H31R	265.99 ± 7.77	0.298	97.61	12.04 (62.5)
	K12M-AAA	270.52 ± 2.20	0.568	99.27	5.98 (31.3)
<i>S. agalactiae</i> ATCC 13813	WT	223.37±3.99			12.58 (62.5)
	AAA-H31R	268.47±5.44	0.001	120.19	24.08 (125)
	K12M-AAA	178.32±6.51	0.001	79.83	95.53 (500)

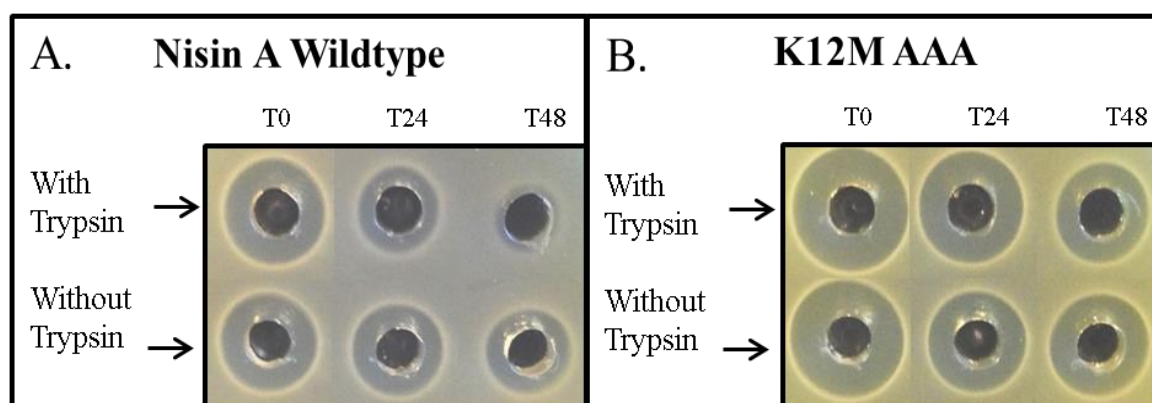
The zone of inhibition is expressed as the area of the zone of inhibition minus the area of the ‘spot’ in mm². MIC: Minimum Inhibitory Concentration

Figure 4.1 A. Nisin A (chymo)trypsin cleavage sites. B. Amino acid changes made across the various nisin A derivatives described in this study



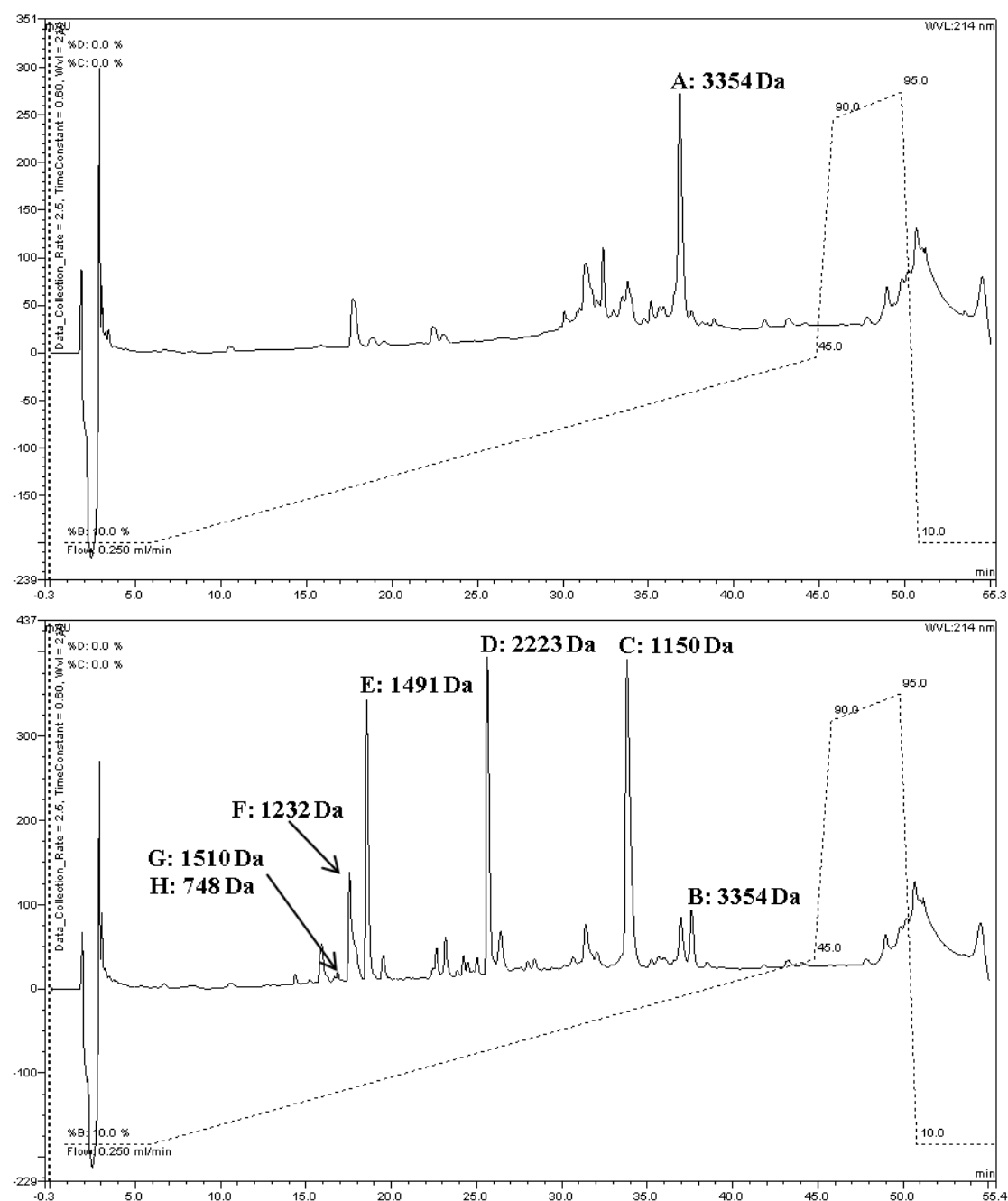
Panel A. Dha – Dehydroalanine; Dhb – Dehydrobutyrine. Arrows denote the (chymo)trypsin cleavage sites within the nisin A peptide. **Panel B** Amino acid changes (in grey) made to produce the bioactive (chymo)trypsin resistant derivatives in the nisin peptide.

Figure 4.2 Enzyme Digest Well Diffusion Assays



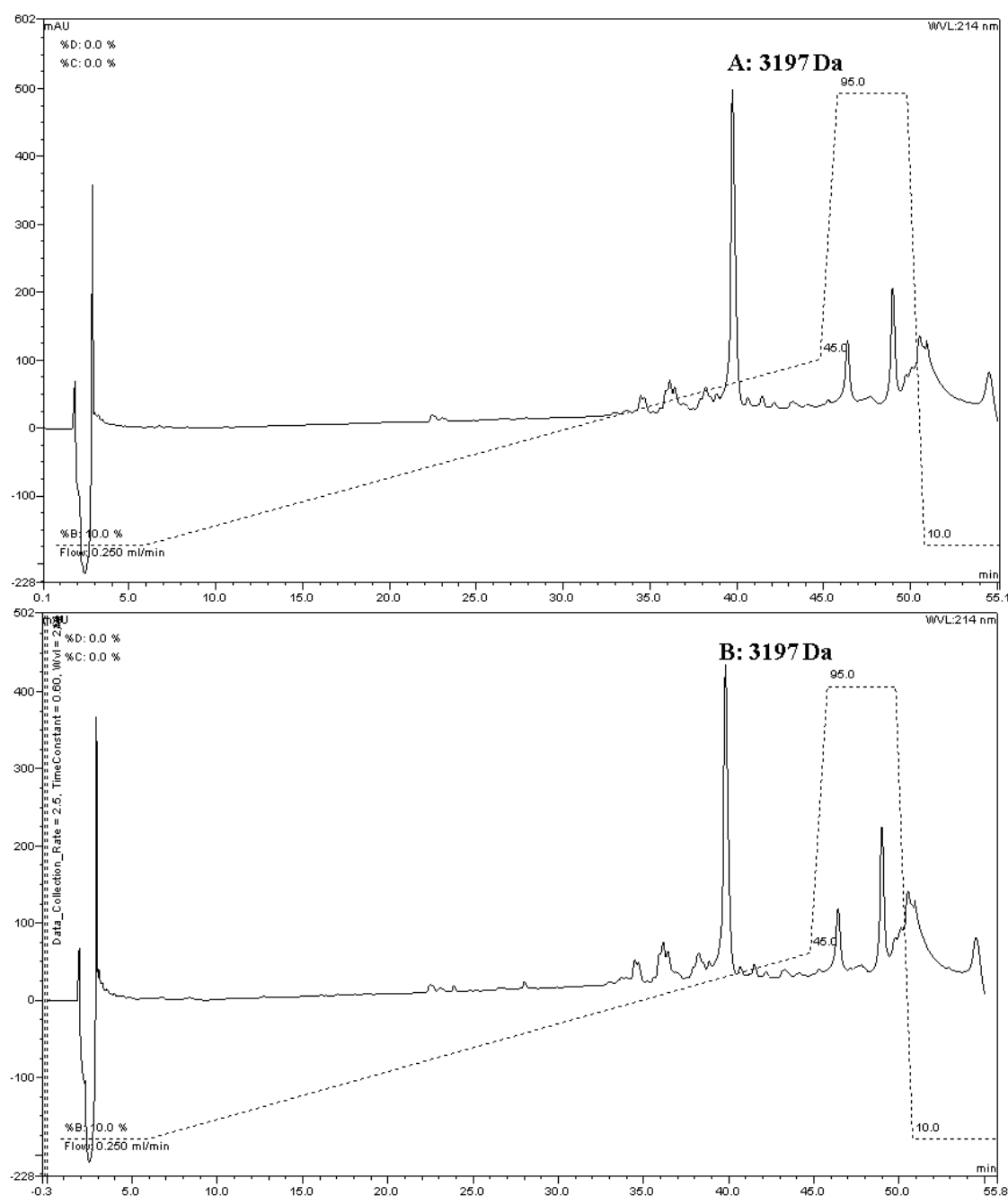
Nisin A wild-type (Panel A.) and K12M-AAA (Panel B.) digests with or without trypsin at T0, T24 hrs and T48hrs.

Figure 4.3 RP-HPLC chromatograms displaying the fragments produced after a 48 hour digestion of nisin A with trypsin



The top chromatogram shows T0, the lower chromatogram shows T48. A: Nisin A WT, B: Nisin A WT, C: Nisin 1-12, D: Trypsin autolysis fragment, E: 21-34, F: 23-34, G: 21-34 (Ser33) and H: 13-20.

Figure 4.4 RP-HPLC chromatograms displaying the fragments produced after a 48 hour digestion of K12M-AAA with trypsin.



The top chromatogram shows T0, the lower chromatogram shows T48. A: K12M-AAA, B: K12M-AAA

Chapter V

Comparison of the efficacy of a variety of nisin derivatives against *Streptococcus agalactiae*

Brian Healy, Colin Hill, Paul D. Cotter and R. Paul Ross

5.1 Abstract

Streptococcus agalactiae is one of a number of pathogens that can cause sub-clinical or clinical bovine mastitis, an inflammation of the mammary glands of infected animals. From an economic viewpoint, bovine mastitis is one of the most important diseases of the dairy industry. *S. agalactiae* is also known to cause neonatal septicaemia in humans. Nisin A, the prototypic member of the lantibiotic (antimicrobial peptides containing lanthionine linkages) class of bacteriocins, exhibits specific activity at the nanomolar level against *S. agalactiae*. We have used molecular bioengineering to create derivatives of nisin that are enhanced with respect to a number of significant traits, such as increased diffusion and/ or specific activity. For this study, a collection of 15 ‘lead’ nisin derivatives were compared with nisin A in order to identify those that most effectively inhibit *S. agalactiae*. Of these, a derivative in which the 3-amino acid hinge region ‘NMK’ was converted to the residues ‘AAA’ proved to be twice as effective as the wild-type in controlling the pathogen in milk over a 24 hour period. Apart from the AAA derivative, both K22T and a variant with an SAA hinge were also more effective than wild-type against *S. agalactiae*. A system was also created allowing the growth of nisin producers in the presence of lactose, which can ultimately facilitate large-scale peptide production in dairy substrates.

5.2 Introduction

Mastitis can be defined as any inflammatory process occurring in the mammary gland (Keane *et al.*, 2013) and is the most common and costly contagious disease affecting dairy farms in the western world (Barkema *et al.*, 2009). A number of Gram-positive and negative pathogens can be the causative agents for the disease, with *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Staphylococcus aureus* and *Escherichia coli* being the most prevalent (Barkema *et al.*, 1998). Two major forms of the disease exist, clinical and subclinical mastitis, with the latter being considerably more difficult to detect and treat. Regardless, the outcome of each form is a decrease in the quality and volume of milk produced by the infected animal (Wu *et al.*, 2007).

An important indication of the presence of sub-clinical mastitis is the Bulk Milk Somatic Cell Count (BMSCC), with the legal accepted limit set at 400 000 cells/ ml (EU Council Directive 92/46/ECC) (More, 2009). Although the average BMSCC count in Ireland is far below this limit (252 000 cfu/ml) (Geary *et al.*, 2014), the number is continually increasing, leading to a negative impact on quality, volume and profit (Teagasc, 2012). This is of great concern due to the importance of the Irish dairy industry to the national economy. Ireland exports 85% of its annual dairy production and there is a 3% annual increase in global demand for dairy products (More, 2009). Secondly, there is a need to increase the volume of milk being produced in the near future. The Department of Agriculture, Food and the Marine (DAFM) has set a target of a 50% increase in the volume of milk produced in Ireland to coincide with the removal of the milk quota in 2015. It is estimated that every reduction in BMSCC by 100 000 cells results in a 142.1 million litre gain (5% of the target) (Geary *et al.*, 2013).

One strategy routinely employed for the treatment of mastitis is the administration of a long-acting antibiotics during the drying off period of the cow (Crispie *et al.*, 2004). It is during this drying off period that non-lactating cows become more susceptible to acquiring new infections (Twomey *et al.*, 2000). However, the emergence of mastitis-associated antibiotic resistant bacteria resulting from this treatment has become an issue. In fact, mastitis treatment is the most common reason for antibiotic use in dairy herds (Rajala-Schultz *et al.*, 2004). As the quantity administered increases, the contamination of milk with antibiotics also becomes an issue due to concerns for human health from antibiotic residues and the inhibition of lactic acid bacteria essential for dairy processes, such as cheese and yoghurt making (Cao *et al.*, 2007).

Bacteriocins, bacterially produced antimicrobial peptides with narrow or broad spectrum host ranges (Cotter *et al.*, 2005), have recently gained greater attention as realistic alternatives to traditional antibiotics. In particular, the Class 1 post-translationally modified lanthionine containing antibiotics, the lantibiotics, have come to prominence due to their ability to inhibit food-associated and antibiotic resistant pathogens (Deegan *et al.*, 2006, Piper *et al.*, 2009). Their attractiveness stems from a number of key characteristics. Many lantibiotics are produced from generally regarded as safe (GRAS) lactic acid bacteria, they are often effective at nanomolar concentrations against clinically relevant pathogens, they are amenable to relatively economic large-scale production and, because they are synthesised through ribosomal pathways, are open to genetic manipulation (Healy *et al.*, 2010). This genetic tolerance to change has been exploited with great success in order to understand the structure/ function of many lantibiotics and, although less common, the appliance of various bioengineering strategies to increase the bioactivity or

functionality of the prototypic lantibiotic nisin has yielded very promising results (Molloy *et al.*, 2012).

S. agalactiae, a leading cause of bovine mastitis, is one of the pathogens against which bioengineered nisin derivatives have been found to be particularly effective. Indeed, *S. agalactiae* was the first Gram-positive pathogen against which a nisin derivative (Nisin K22T- a nisin A derivative containing a threonine rather than a lysine at position 22) with enhanced activity was identified (Field *et al.*, 2008). This breakthrough led to a focus on the central three amino acid hinge region of the nisin peptide (N20, M21 and K22) and the subsequent use of both site specific and site saturation mutagenesis techniques to generate other enhanced nisin derivatives that are altered in this region; i.e. N20P, M21V and K22S (Field *et al.*, 2008). This research provided the impetus for even further studies which lead to the creation of additional enhanced nisin derivatives (Field *et al.*, 2008, Field *et al.*, 2012, Rouse *et al.*, 2012, Healy *et al.*, 2013, Molloy *et al.*, 2013).

For this study, a collection of these lead nisin derivatives, and their producers, were tested in order to identify those that most effectively control the growth of *S. agalactiae*. Nisin A and derivatives were expressed in the *Lactococcus lactis* Lac⁻/Prt⁻ NZ9800 (Kuipers *et al.*, 1993) background, a derivative of the nisin A producer *L. lactis* NZ9700 with a 4 bp deletion in the *nisA* structural gene. Nisin production by this strain is restored through complementation of NZ9800 with a functioning *nisA* gene, or derivative, on the plasmid pCI372 (pDF05) (Field *et al.*, 2008). In total, 15 derivatives were tested and ranked according to the levels of bioactivity displayed against *S. agalactiae* ATCC13813. Six derivatives were brought forward for further study in the form of miniature milk trials. Finally, to facilitate the growth of the various producers in a dairy-based substrate to facilitate

peptide generation, a variety of strains were created that possessed both the ability to produce nisin derivatives and to utilise lactose and degrade casein, as a consequence of containing the plasmid pLP712 (Lac⁺ Pro⁺) (Gasson, 1983).

5.3 Materials and Methods

5.3.1 Bacterial strains and growth conditions

The strains used in this study are listed in Table 5.1. *L. lactis* NZ9800 was grown in M17 agar and broth (Oxoid) supplemented with 0.5% glucose. *L. lactis* MG1614 pLP712 was grown in M17 agar and broth supplemented with 0.5% lactose. Both cultures were grown at 30°C for 18 hrs. *S. agalactiae* ATCC13813 was grown on Tryptic Soy Broth (TSB) (Merck) supplemented with 0.6% (w/v) Yeast Extract (Merck) and incubated at 37°C for 18hrs. Where required, chloramphenicol (Sigma-Aldrich) was added at 10 µg/ml and rifampicin (Sigma-Aldrich) was added at 50 µg/ml.

5.3.2 Rifampicin tagging of *S. agalactiae* ATCC13813

S. agalactiae ATCC 13813 was grown overnight at 37°C in a 10 ml volume with constant shaking. The culture was centrifuged at 7000 rpm for 10 min. The supernatant was decanted with the remaining pellet resuspended in 1 ml of Phosphate Buffered Saline (PBS). A 100 µl volume was spread plate onto TSB-YE agar containing rifampicin (Sigma) concentrations ranging from 10 µg/ml to 200 µg/ml and incubated overnight at 37°C. Colonies were present at a highest concentration of 100 µg/ml and subsequently restreaked onto TSB-YE agar Rif 100 µg/ml. An isolated colony was passaged 10 times in TSB-YE broth without rifampicin and plated on TSB-YE Rif 100 µg/ml to determine that the tagging was stable. Subsequent selection was made on TSB-YE Rif 50 µg/ml.

5.3.3 Bioactivity Assays

Cultures of nisin A wild-type and the derivatives were grown overnight, with a 1% subculture for 8 hours followed by a second 1% subculture for 16 hr. At each step 10 ng/ml of nisin A was added to the 10 ml GM17 broth. 3 µl of each culture was 'spotted' onto GM17 agar, allowed to dry and incubated for 16 hr at 30°C. The remaining sample was centrifuged at 5000 rpm for 10 min, the supernatant carefully removed and heated to 80°C for 10 min and stored at -20°C until required. The spotted plates were UV treated for 20 min in order to kill the producer and overlaid with *S. agalactiae* ATCC 13813 Rif^R (0.5% v/v) in TSB-YE 0.75% agar and incubated at 37°C for 16 hr. Well diffusion assays were carried out in TSB-YE 1% agar. 70 ml volumes of this agar were inoculated with 0.5% (v/v) of an overnight culture of *S. agalactiae* ATCC 13813 Rif^R. Wells were bored using an inverted glass Pasteur pipette and 70 µl of the cell-free supernatant (CFS) from above added. Before incubation (37°C for 16 hr) the plates were refrigerated for three hours so as to allow diffusion of the peptides before commencement of indicator growth.

5.3.4 Determination of Antimicrobial Activity in Milk

In order to measure the level of antagonism of the CFS from nisin A wild-type and the derivatives in an applied setting, a miniature milk trail was completed in triplicate. 1.4 mls of commercially available milk (Avonmore) was inoculated with *S. agalactiae* ATCC 13813 Rif^R at 10⁶ cfu/ml in 24-well tissue culture flasks (Sarstedt). 0.6 mls of CFS was added from each of the nisin derivatives brought forward was added and the plates incubated at 37°C for 24 hrs. Counts were taken at T0 and T24.

5.3.5 Creation of *L. lactis* NZ9800 pLP712

L. lactis MG1614 pLP712 (donor) was grown to a final volume of 30 mls in LM17 broth and *L. lactis* NZ9800 pDF05 (recipient) was grown to a final volume of 10 mls in GM17 broth with 10 µg/ml chloramphenicol. Both strains were washed in fresh media without antibiotic. 10 mls of the recipient was passed through a 0.45 µm filter followed by 500 µl of 800 µg/ml chymotrypsin and left for 5 minutes in order to hydrolyse any residual nisin. 30 mls of the donor was next passed through the same filter until dry. The filter was next removed and placed top-side down onto GM17 agar without chloramphenicol and incubated for 24 hrs at 30°C. The filter and the agar underneath was removed, sliced and placed in 10 mls of ¼ strength ringer solution and vortexed for 5 mins or until the turbidity of the ringer solution increased. 10 µl of this solution was streaked onto lactose indicator agar (LIA) Cm¹⁰. Desired transconjugates (*L. lactis* NZ9800 pDF05 pLP712) were selected due to their ability to ferment lactose, and therefore appear as yellow, and grow in the presence of chloramphenicol. pDF05 was cured from the strain through continual passaging of the strain in LM17 broth without chloramphenicol until sensitivity to the antibiotic was apparent.

5.4 Results

15 previously generated nisin derivatives were selected to compare their activity against *S. agalactiae*. The rationale used to select these nisin derivatives was as follows. Firstly, derivatives were chosen which had been reported to have particularly enhanced features during the course of previous studies. These were N20P, M21V, K22T and K22S (Field *et al.*, 2008), SVA (Rouse *et al.*, 2012), S29A (Field *et al.*, 2012), K12A (Molloy *et al.*, 2013), hinge ‘SAA’ and hinge ‘AAA’ (hereafter SAA and AAA) (Healy *et al.*, 2013). Secondly, a number of additional derivatives that have not been investigated to the same degree, but which possess potential anti-*S. agalactiae* bioactivity were also included. These were peptides in which the hinge regions had been altered to SLS, AAK, SMT, NAI or QVQ (Healy *et al.*, 2013). Finally, a derivative containing a NAT hinge was included it has emerged as a peptide of interest during various screening studies (unpublished). For this study, enhanced bioactivity is used to describe a change that results in greater inhibition regardless of whether this is due to increased specific activity, enhanced production levels and/ or other physico-chemical properties (such as enhanced diffusion). In order to determine the most promising candidates from this pool as potential bioprotectants, the deferred antagonism assay and well diffusion assays were employed as a measure of bioactivity against *S. agalactiae* ATCC 13813 Rif^R. As the focus of this study was the identification of strains that most effectively control the pathogen, and not the characterisation of their conferred benefit, general bioactivity was used as a measure of potential. All peptides were produced by derivatives of *L. lactis* NZ9800.

The results from well diffusions assays (Table 5.2) showed that cell free supernatant (CFS) containing nisin A, AAA, K22T, K22S, SAA and K12A most

effectively inhibited the pathogen. However, of these, CFS containing AAA, K22T, K22S, and SAA produced zones that did not differ significantly from that of the control while K12A produced a significantly smaller zone. The results from the deferred antagonism assay (Table 5.3) clearly demonstrate the enhanced bioactivity of the majority of derivatives relative to the control, nisin A-producing strain. The five strains with greatest bioactivity (all p-values <0.05) were those producing the AAA, NAT, SAA, K22S and K22T derivatives. When the area of inhibition is expressed as a percentage of that generated by the control, AAA shows the greatest enhancement (70.54% increase) followed by NAT (51.94% increase), SAA (51.63% increase), K22S (49.68% increase) and K22T (46.60% increase). Another five strains showed relative increases ranging from 42.05% (QVQ) down to 27.09% (SMT). Only N20P displayed a zone significantly lower than that generated by the control. Ultimately, 6 derivatives were brought forward to the miniature milk assay (Table 5.4); AAA, SAA, NAT, K22T, K22S and K12A.

Miniature milk trials were performed to ensure that the derivatives retain activity in a dairy setting and used commercially available, pasteurized and homogenised full fat milk. *S. agalactiae* ATCC 13813 Rif^R was inoculated at 5.1 X 10⁶ cfu/ml of milk. 600 µl of nisin-containing CFS was added to 1400 µl of milk and incubated at 37°C for 24 hrs. The procedure was carried out in triplicate with plate counts carried out at T0 and T24. Milk to which neither the pathogen nor CFS was added was used as a negative control and milk inoculated with *S. agalactiae* ATCC 13813 Rif^R was used as a positive control. CFS from the non-nisin producer NZ9800 was also used as a control and was found to have no significant impact on the growth of *S. agalactiae*. The results of this trial were expressed in terms of log reduction of growth relative to that observed in the presence of CFS from NZ9800 after T24

(Table 5.4) The CFS from the AAA derivative producer brought about a log reduction of 2.30 (p-value 0.032), the largest reduction of any of the samples tested, being twice as effective as wild-type CFS (1.15 log reduction). K22T also brought about a greater than 2 log reduction (2.10, $p = 0.036$). SAA, WT and K12A all displayed a greater than 1 log reduction while K22S and NAT proved to be the least effective of those tested, failing to give a greater than 1 log reduction. Following on from these results, it was clear that AAA and K22T showed the greatest potential as biologically produced inhibitors of *S. agalactiae* in a milk environment.

The creation of forms of these nisin derivative-producing starter cultures that could grow in dairy substrates was the final aim of this study. This was achieved through the conjugation of the plasmid pLP712 from *L. lactis* MG1614 to *L. lactis* NZ9800 through a filter-mating procedure in order to provide the latter strain with the genes necessary for lactose catabolism and casein proteolysis. The transconjugant generated, *L. lactis* NZ9800 pLP712, was grown in 10% total solids Reconstituted Skimmed Milk (RSM) to demonstrate its ability to ferment lactose at 30°C for 16 hrs with subsequent clotting of the milk. Lantibiotic production was reintroduced by transformation of the *nisA* structural gene or a bioengineered equivalent on pCI372 into the transconjugant. The production of the nisin derivatives AAA, K22T and SAA by such strains was demonstrated by the deferred antagonism assay with the producer ‘spotted’ onto both GM17 and LM17 agar with *S. agalactiae* as the indicator. MALDI TOF mass spectrometric analysis was used to confirm the production of the relevant peptides (data not shown). These strains can be grown on cheap lactose-containing substrates to facilitate large scale peptide generation.

5.5 Discussion

LAB-associated bacteriocins, and in particular lantibiotics, are an attractive option for the control of pathogenic and spoilage organisms in the food industry. This is due in part to the long and safe history of nisin use by the food industry (Cotter *et al.*, 2005) and the variety of ways in which bacteriocins can be incorporated into foods. Indeed, bacteriocins can be introduced through direct addition as a preservative, as an ingredient in the form of a dried fermentate or through *in situ* production. In the case of nisin, all three methods of delivery have been exploited in commercial products (Healy *et al.*, 2010, Elsser-Gravesen and Elsser-Gravesen, 2013). For this study, the anti-*S. agalactiae* bioactivity of a number of nisin derivatives was assessed and a system developed that could potentially be employed to facilitate their production on cheap lactose-based substrates.

Lantibiotics, including nisin and lacticin 3147, have been shown to be very effective against a number of mastitis associated pathogens (Ryan *et al.*, 1998, Field *et al.*, 2008) *in vitro*. This activity has also translated well to a number of potential therapeutic applications to treat mastitis infections as either purified forms or through the introduction of the live culture. For example, lacticin 3147 has proved effective in treating *S. dysgalactiae* and *Staph. aureus* when introduced by means of an intramammary teat seal formulation (Ryan *et al.*, 1999, Twomey *et al.*, 2000, Crispie *et al.*, 2008) and against the pathogens and *Streptococcus uberis* when employed in the form of a teat dip (Klostermann *et al.*, 2010). Intramammary infusions (IMI's) of nisin have also displayed a therapeutic effect in the treatment of both subclinical and clinical mastitis (Cao *et al.*, 2007, Wu *et al.*, 2007). The use of lantibiotics as an alternative to antibiotics in the treatment of mastitis also could also

address issues and concerns regarding the emergence of multidrug resistant human pathogens due to exposure to antibiotic residues in milk (Cao *et al.*, 2007). It is therefore not surprising that nisin has been commercialised as a teat wipe, Wipe Out®, and as the active ingredient in an IMI product, Mast Out® (both produced by ImmuCell).

The results of this study demonstrate that the nisin ‘hinge’ derivative, AAA, is a leading candidate that merits further investigations as either a bio-preservative or as an alternative to antibiotics in prophylactic anti-*S. agalactiae* mastitis treatments. In all three assays employed here, this derivative, in which the all three of the naturally occurring amino acid residues within the ‘hinge’ were replaced with alanines, displayed activity at levels greater or similar to that of the parental producer. Of the other derivatives, the activities of both K22T and SAA with respect to pathogen inhibition in milk were also impressive. As discussed previously (Field *et al.*, 2008, Healy *et al.*, 2013), the hinge region of nisin has proved to be extremely important in respect to the creation of enhanced derivatives with its flexibility in terms of both the residues tolerated within and in terms of overall mode of action of the peptide. This was again apparent in this study.

It was noticeable that the relative ability of the derivatives to control the pathogen varied depending on the approach used. Of the fifteen strains tested, thirteen displayed a zone of inhibition larger than the wild-type when ‘spotted’ while only CFS containing nisin AAA produced a zone larger (albeit non-significantly) than wild-type *via* the well diffusion approach. This suggests that production of the candidate derivatives by Lac⁺ Prt⁺ lactococci *in situ* in the teat canal in order to control *S. agalactiae* merits consideration. Having Lac⁺ Prt⁺ producers of these derivatives also provides the opportunity to use cheap protein-rich substrates for

large scale peptide production. One means of generating such a strain would be through a homologous recombination event in an already available nisin wild-type producing culture in order to ‘swap’ the *nisA* structural gene with that of the derivative of interest. This technique can be time consuming and relatively difficult to achieve and so, before initiating such a strategy, we first used an alternate strategy by creating a transconjugant of the *L. lactis* strain NZ9800 containing a plasmid which confers onto the host the genes necessary for lactose catabolism and proteolysis (pLP712). Although lacking the rapid acidification rates of the more established starter cultures, this strain does provide a simpler and quicker means to assess the bioactivity and peptide producing capacity of nisin derivative producers on milk-based substrates. This system provides a means whereby not only the derivatives assessed here but any potential designer lantibiotics could be assessed in a more time efficient manner through the production of fermentates/ dairy products using relatively inexpensive whey-based substrates to produce large quantities of powders. The producers of greatest value could then be created in a non-GM manner through a homologous recombination-based approach and, potentially, also introduced into the lactose rich environment of the mammary gland as an IMI.

The treatment of mastitis through the use of nisin derivatives is potentially very worthwhile due to the activity of many of these antimicrobial peptides against many of the Gram-positive causative agents (Field *et al.*, 2010). In this study, three derivatives of nisin were identified (AAA, K22T and SAA) which showed enhanced activity when compared to nisin in a CFS form against *S. agalactiae* in a miniature milk trail. As all three have also demonstrated enhanced activity against *Staph. aureus* previously (Field *et al.*, 2010, Healy *et al.*, 2013), their inclusion for any potential trials to assess the therapeutic effectiveness of nisin derivatives as an

alternative to antibiotics would be merited. Further investigations into the addition of these derivatives as bio-preservatives in dairy preparations either as an additive or by production as a starter may also prove beneficial.

5.6 Acknowledgements

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5.8 Tables and Figures

Table 5.1 Strains used in this study

Strains	Characteristic	Reference/Source
<i>L. lactis</i> NZ9800	<i>L. lactis</i> NZ9700 $\Delta nlsA$	Kuipers <i>et al.</i> (1993)
<i>L. lactis</i> NZ9800 pDF05	Wild-type nisin A producer	Field <i>et al.</i> (2008)
<i>S. agalactiae</i> ATCC 13813 Rif ^R	Rifampicin tagged indicator strain	This study
<i>L. lactis</i> MG1614 pLP712	Lactococcal donor of Lac ⁺ Pro ⁺ pLP712	O'Sullivan <i>et al.</i> (1998)
<i>L. lactis</i> NZ9800 pLP712	Lac ⁺ Pro ⁺ <i>L. lactis</i> NZ9800	This study

Table 5.2 Well Diffusion Assay using Cell Free Supernatant

Indicator	Producer	Well Diffusion			
		Average Area mm ²	As % of WT	p-value	Std. Dev.
<i>S. agalactiae</i> ATCC 13813 Rif ^R	WT	209.49			9.02
	AAA	225.17	107.49	0.13	10.71
	K22T	206.89	98.76	0.69	5.29
	K22S	204.35	97.55	0.46	5.87
	SAA	204.35	97.55	0.46	5.87
	K12A	190.27	90.83	0.05	7.49
	M21V	184.74	88.19	0.07	14.05
	NAT	178.24	85.09	0.01	6.87
	S29A	168.25	80.32	0.01	12.52
	NAI	165.94	79.21	0.01	11.45
	AAK	156.08	74.51	0.00	3.85
	SVA	148.09	70.69	0.00	5.44
	SMT	147.38	70.36	0.00	6.45
	QVQ	145.34	69.38	0.00	11.19
	SLS	128.61	61.40	0.00	0.00
	N20P	109.37	52.21	0.00	6.64
	NZ9800	20.69	9.88	0.00	0.47

The zone of inhibition is expressed as the area of the zone of inhibition minus the area of the ‘well’ in mm²

Table 5.3 Deferred Antagonism Results

Indicator	Producer	Deferred Antagonism			
		Average Area mm ²	As % of WT	p-value	Std. Dev.
<i>S. agalactiae</i> ATCC 13813 Rif ^R	WT	279.95			10.48
	AAA	477.44	170.54	0.00	14.23
	NAT	425.37	151.94	0.00	7.06
	SAA	424.50	151.63	0.00	11.88
	K22S	418.81	149.60	0.00	21.27
	K22T	410.40	146.60	0.00	16.47
	QVQ	397.67	142.05	0.01	24.86
	K12A	390.92	139.64	0.02	31.69
	SVA	381.53	136.29	0.01	22.41
	NAI	356.6	127.38	0.07	39.91
	SMT	355.78	127.09	0.00	13.12
	M21V	342.11	122.20	0.07	32.01
	S29A	325.58	116.30	0.03	18.18
	SLS	288.61	103.09	0.61	24.21
	AAK	272.00	97.16	0.48	14.04
	N20P	202.57	72.36	0.02	23.00
	NZ9800	0.00	0.00	0.00	0.00

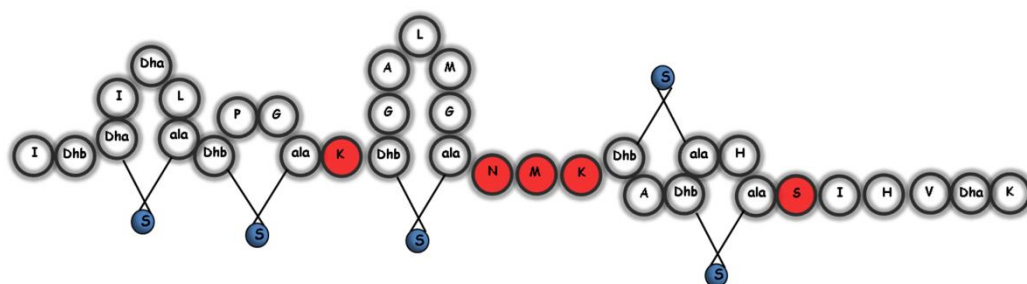
The zone of inhibition is expressed as the area of the zone of inhibition minus the area of the ‘colony’ in mm²

Table 5.4 Miniature Milk Trial using Cell Free Supernatant

Indicator	Producer	Mean Log cfu/ml \pm SEM at T24	p-Value relative to NZ9800	Log reduction relative to NZ9800
<i>S. agalactiae</i> ATCC 13813 Rif ^R	NZ9800 (Control)	9.44 \pm 0.05		
	AAA	7.14 \pm 0.43	0.032	-2.30
	K22T	7.33 \pm 0.42	0.036	-2.10
	SAA	7.77 \pm 0.43	0.061	-1.66
	WT	8.18 \pm 0.36	0.069	-1.25
	K12A	8.29 \pm 0.33	0.072	-1.15
	K22S	8.48 \pm 0.21	0.041	-0.96
	NAT	8.58 \pm 0.25	0.069	-0.85
	Positive	9.26 \pm 0.03	0.073	
	Negative	$\leq 0.15 \pm 0.00$		

T0 count 6.70 ± 0.02 . SEM: Standard error of the mean. Those giving a log reduction greater than wild-type are emboldened.

Figure 5. 1 Nisin A amino acid structure



Dha – Dehydroalanine; Dhb – Dehydrobutyrine. Areas in red denote locations of derivatives described in this study.

Summary

Lantibiotics are ribosomally produced antimicrobial peptides (Sahl *et al.*, 1995). The mature lantibiotic peptide structure is the result of a number of post-translational modifications. The unmodified and inactive prepeptide is first exposed to the actions of a dehydratase enzyme (generally referred to as LanB) which leads to the formation of 2,3-dehydroalanine and 2,3-dehydrobutyrine from specific serine and threonine amino acid residues, respectively. A number of these residues are subsequently coupled with the thiol groups of neighbouring cysteine residues resulting in the formation of the eponymous (methyl)lanthionine bridges. The peptide is transported to the cell surface through the action of an ABC transport system where it becomes active following cleavage of its leader sequence.

This thesis focuses on two of the most widely studied members of this class of bacteriocins, the single component peptide nisin A and the two peptide lactacin 3147. The mode of action of both is similar in that it involves binding to the essential cell wall precursor, lipid II, followed by pore formation. Nisin A acts through the combined action of its N and C-termini through an intra-molecular conformational change facilitated by the flexible three amino acid 'hinge' region (Breukink *et al.*, 1997, Wiedemann *et al.*, 2001). For lactacin 3147, a synergistic interaction between both peptide components is facilitated through the formation of a complex between the α peptide and lipid II, which then recruits the β peptide and leads to rapid cell death (McAuliffe *et al.*, 1998, Wiedemann *et al.*, 2006).

In Chapter 1, the lantibiotic-associated post-translational modifications are reviewed in greater detail, while an overview of the various lantibiotic classification systems is provided. This review also summarises that which is known about the

structure and mode of action of the lantibiotic subgroups, the ways in which these peptides are used in the food industry, the current and potential lantibiotic uses in medical applications or as veterinary therapeutics and the bioinformatics-based screening techniques that are emerging as alternative approaches to discovering novel lantibiotics.

In Chapter 2, we investigated the *in situ* production of lacticin 3147 by the starter culture *Lactococcus lactis* DPC3147 during the manufacture of a smear-ripening raw milk cheese with a view to controlling the growth of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). *MAP*, an extremely fastidious bacterium, is the causative agent of Johne's disease (Cocito *et al.*, 1994), a chronic enteritis of the small intestine primarily affecting ruminants and a disease of economic importance to dairy industries. Similarities in disease pathologies have led to suggestions of a link between *MAP* and Crohn's disease, although the existence of such a relationship is not clear. Due to the robust nature of the microorganism in terms of its innate resistance to various physical treatments, such as pasteurization and disinfectant agents such as chlorine, contaminated milk from Johne's diseased dairy cattle could possibly offer a route of entry into the human food chain (Rowe & Grant, 2006). In a previous study (Carroll *et al.*, 2010), lacticin 3147 was shown to display *in vitro* activity against *MAP* (MIC₉₀, 15 mg/l) while nisin was at least four times less effective (MIC₉₀, >60 mg/l). While our study demonstrated a limited reduction in *MAP* numbers, as determined by culture based analysis, at the end of the ripening period, this was attributed to the cheese making process rather than the presence of lacticin 3147. A real-time PCR strategy was implemented which provided results that were consistent with those provided by plate counts. This rapid alternative to detecting/ quantifying *MAP* is noteworthy as the incubation time required for the

visualization of *MAP* on synthetic media is normally six weeks and was further complicated by the levels of background flora associated with this type of cheese.

In Chapter 3, the gene encoded nature of lantibiotics was exploited with a view to the creation of derivatives with enhanced (bio)activity. The focus here switched to nisin A and in particular the three amino acid ‘hinge’ region consisting of asparagine, methionine and lysine residues at positions 20, 21 and 22. This region allows for the conformational realignment of the peptide once a complex is formed with lipid II, facilitating the permeabilization of the cell membrane by the C-terminus. Studies by Field *et al.* (2008) had demonstrated that the substitution of the lysine at position 22 for a threonine (K22T) residue displayed enhanced activity against *Streptococcus agalactiae*. Further investigations revealed the relative tolerance of this region to change and lead to the creation of three further enhanced nisin derivatives; N20P, M21V and K22S. We undertook a complete randomisation of the hinge region whereby a derivative bank was produced using indiscriminate site saturation through the use of degenerate primers. A bank of 12,000 putative producers was screened against a fast growing mycobacteria species, *Mycobacterium smegmatis*, as well as clinical pathogens (*Staphylococcus aureus* and *S. agalactiae*) and a nisin sensitive indicator (*L. lactis* HP). From this bank, a total 23 enhanced derivatives were identified. The frequency at which alanine was present at each position prompted the creation of a nisin derivative whereby the ‘hinge’ consisted of three alanines. Characterisation of this derivative revealed that this change enhanced the bioactivity of the producing strain while the specific activity of the purified form was twice as potent as nisin A against *S. agalactiae*. An additional derivative was created based on the observation that a number of the aforementioned derivatives possessed a serine at position 20. This derivative, SAA, also displayed enhanced

bioactivity against all indicators. This study once again established the ‘hinge’ as a productive target for bioengineering and specifically illustrated the benefits of incorporating small chiral amino acids into this site. This approach led to the creation of two enhanced nisin A derivatives through a rational approach for the first time.

Improvements in terms of the functionality of the nisin A peptide were demonstrated in Chapter 4. A major hurdle in the utility of nisin in a clinical setting is due to its sensitivity to proteolytic degradation during transit through the gastrointestinal tract. One such protease is trypsin, which normally cleaves after lysine and arginine residues. However, it was recently found that the asparagine amino acid residue at position 20 is also subject to the endopeptidase activity of this enzyme (Slootweg *et al.*, 2013). While the lanthionine bridges confer the mature peptide with a natural partial trypsin resistance, unmodified lysines at positions 12 and 22 and the asparagine at position 20 are still vulnerable. As the ‘hinge’ region of the AAA derivative from Chapter 3 no longer contains these residues, position 12 (previously shown to be receptive to change (Molloy *et al.*, 2013)), is the only remaining trypsin sensitive region in nisin AAA. Through the implementation of site saturation mutagenesis, a mutant was identified which displayed enhanced bioactivity compared with the wild-type nisin producer, while also gaining resistance to trypsin digestion (as confirmed through RP-HPLC and mass spectrometric analysis). During the screening aspect of this study a number of putative chymotrypsin derivatives were also uncovered which will merit more in-depth characterisation in the future.

Chapter 5 of this thesis highlights the potential of a number of nisin A derivatives against the mastitis associated pathogen *S. agalactiae*. Lantibiotics may provide an alternative means of controlling this bacterium as it is extremely sensitive to nisin. Notably, as a result of the evolution of nisin bioengineering strategies, a

large bank of anti-*S. agalactiae* derivatives has been generated. In this study, 15 of these derivatives were assessed in order to identify those which most effectively inhibit the pathogen in a milk matrix. Again, nisin AAA, along with K22T and SAA, emerged from this study as lead candidates. A system was also developed to facilitate large-scale peptide production of these peptides in dairy substrates with a view to the manufacture of mastitis therapeutics as either fermentates or powdered ingredients.

In conclusion, lantibiotics are attractive alternatives to antibiotics for a number of reasons. This includes their potency against clinically relevant pathogens and their amenability to bioengineering. In this thesis a number of lantibiotic derivatives of interest were discovered. These add to our understanding of fundamental lantibiotic biology and strengthen the case for a greater role for these antimicrobial peptides in a clinical and food setting.

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“Research is what I'm doing when I don't know what I'm doing”.

Wernher von Braun