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Bioengineering Nisin to overcome the Nisin Resistance Protein

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Running Head: A nisin derivative insensitive to NSR

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Summary

The emergence and dissemination of antibiotic resistant bacteria is a major medical challenge. Lantibiotics are highly modified bacterially produced antimicrobial peptides that have attracted considerable interest as alternatives or adjuncts to existing antibiotics. Nisin, the most widely studied and commercially exploited lantibiotic, exhibits high efficacy against many pathogens. However, some clinically relevant bacteria express highly specific membrane-associated nisin resistance proteins. One notable example is the nisin resistance protein (NSR) that acts by cleaving the peptide bond between ring E and the adjacent serine 29, resulting in a truncated peptide with significantly less activity. We utilised a complete bank of bioengineered nisin (nisin A) producers in which the serine 29 residue has been replaced with every alternative amino acid. The nisin A S29P derivative was found to be as active as nisin A against a variety of bacterial targets but, crucially, exhibited a 20-fold increase in specific activity against a strain expressing the nisin resistance protein. Another derivative, nisin PV, exhibited similar properties but was much less prone to oxidation. This version of nisin with enhanced resistance to specific resistance mechanisms could prove useful in the fight against antibiotic resistant pathogens.

Key Words: bacterial resistance, nisin resistance protein, antimicrobial peptide, nisin, lantibiotic, bacteriocin.

Introduction

The pharmaceutical industry and health care systems have been combating antibiotic-resistant strains of bacteria for more than 60 years. The development of novel potent antimicrobial compounds to address clinical problems caused by multi-drug resistant bacteria remains a significant challenge. There is a need for new structural classes of antibiotics, or derivatives of existing antimicrobials, that can overcome these resistance mechanisms. In this regard, a sub-group of antimicrobial peptides termed lantibiotics make excellent candidates as a result of their high potency, low toxicity and the fact that they can be bioengineered (Cotter *et al.*, 2013, Knerr & van der Donk, 2012). Lantibiotics are bacteriocins produced by a large number of Gram-positive bacteria. Importantly, several lantibiotics including mutacin 1140, microbisporicin (NAI-107), actagardine (NVB-302 and duramycin (moli1901) have demonstrated excellent *in vivo* activities and have progressed towards clinical evaluation for

the treatment of life-threatening diseases (Dawson & Scott, 2012, Sandiford, 2015, Ongey *et al.*, 2017). Indeed, a range of advantageous features that includes a lack of emerging resistance, protease and heat stability as well as synergistic activity with other antimicrobials make them suitable for use in human and veterinary medicine (Dischinger *et al.*, 2014).

The most extensively characterised and utilised lantibiotic is nisin. This polycyclic, 34 amino acid peptide (Fig. 1) exhibits antibacterial activity against a wide range of Gram-positive bacteria and a small number of Gram-negative bacteria including *Escherichia coli*, *Helicobacter pylori* and *Acinetobacter baumannii* (Kim *et al.*, 2003, Kuwano *et al.*, 2005, Li *et al.*, 2018). Nisin exerts its antimicrobial activity both by pore formation and by inhibition of cell wall synthesis through specific binding to lipid II, an essential precursor of the bacterial cell wall (Breukink *et al.*, 1999, Hsu *et al.*, 2004, Wiedemann *et al.*, 2001). Notably, microbes have not developed any significant spontaneous resistance to nisin despite its widespread use in the food industry (Kuipers *et al.*, 1993). However, it is likely that its broader use for clinical and/or veterinary purposes could select for resistant strains. Indeed, some bacterial strains have been reported to be innately resistant to nisin *via* various mechanisms such as cell wall modification, biofilm formation or the expression of resistance proteins (Draper *et al.*, 2015).

One example of the latter is the nisin resistance protein (NSR) which was identified decades ago in some strains of *Lactococcus lactis* and which provides resistance through the proteolytic cleavage of nisin (Froseth & McKay, 1991). The ability of NSR to cleave nisin is notable given previous reports of lantibiotics exhibiting high resistance to protease cleavage (Twomey *et al.*, 2002). More detailed analyses have revealed that NSR can proteolytically inactivate nisin by cleaving the peptide bond between $MeLan^{28}$ and Ser^{29} (Fig.1) resulting in a truncated nisin ($nisin^{1–28}$). Moreover, the truncated $nisin^{1–28}$ mutant exhibits a markedly reduced affinity for the bacterial membrane, a significantly reduced effectiveness in pore

formation in the target membrane and up to a 100-fold decrease in bactericidal activity (Sun *et al.*, 2009). Additionally, the first three-dimensional structure of a nisin resistance protein from *Streptococcus agalactiae* COH1 (*Sa*NSR) has been elucidated (Khosa *et al.*, 2016a).

Several lantibiotic bioengineering strategies have been described that provide examples of how peptide functionality can be modified significantly by changing as little as one residue (Field *et al.*, 2008, Islam *et al.*, 2009, Boakes *et al.*, 2012, Ross *et al.*, 2012, Chen *et al.*, 2013). In this study we applied the dual approach of site-specific and site-saturation mutagenesis techniques to create nisin derivatives possessing enhanced resistance to proteolytic cleavage by NSR whilst also maintaining antimicrobial activity. This involved the alteration of residue Serine 29 alone, as well as Serine 29 and Isoleucine 30 in combination. This process led to the ultimate identification and detailed characterisation of one such derivative that is invulnerable to NSR.

Results

Identification of nisin derivatives with enhanced bioactivity against *L. lactis* subsp. diacetylactis DRC3 expressing the nisin resistance protein (NSR)

The importance of serine 29 to the antimicrobial activity of nisin has been highlighted on several occasions (Sun *et al.*, 2009, Field *et al.*, 2012, Chan *et al.*, 1996). Here, a complete collection of strains producing nisin with all 19 possible alternatives at position 29, nisin S29X, was screened using deferred antagonism agar diffusion assays to identify those that display enhanced potency against the target NSR-producing *L. lactis* subsp. *lactis* biovar diacetylactis DRC3 (*L. lactis* DRC3). Candidates of interest were distinguished by zones of inhibition that were larger and more distinct than those generated by the nisin A producing control against the target strain. Notably, only the variant with a proline substitution (S29P)

displayed superior bioactivity against *L. lactis* DRC3 (Fig. 2). This contrasts somewhat with previous findings that this strain exhibited modestly reduced bioactivity against another lactococcal indicator *L. lactis* HP (Field *et al.*, 2012). However, it should be noted that HP is not known to possess any nisin resistance determinants.

Given the clearly enhanced potency against *L. lactis* DRC3, the S29P derivative was selected for purification to allow quantification of its specific activity by MIC determination. The standard RP-HPLC protocol yielded a single peak (data not shown) which, on the basis of mass spectrometry, contained the S29P peptide but also a significant quantity of the peptide in an oxidized form (+16 Da). Following optimisation of the solvent gradient, the two forms were successfully separated (Supplementary Fig. 1A), which enabled purified S29P peptide to be obtained, albeit at a lower yield compared to wild type nisin A. In parallel to these studies, a second site-saturation mutagenesis-based strategy was employed in a bid to expand the repertoire of nisin derivatives in the region around the critical serine 29 to potentially identify derivatives with further improved bioactivity. Specifically, a bank of strains producing derivatives was created in which residues Ser29 and Ile30 were randomised in combination, to obtain potentially all other natural amino acid substitutions (i.e. 20 X 20 = 400). The resultant bank of approximately 2,000 individual producers (i.e. 5x coverage) was screened using deferred antagonism agar diffusion assays against *L. lactis* DRC3. From this screen, 8 enhanced producers were selected for further investigation. Mass spectrometric analysis of the peptide produced by each strain established that 5 of these (those producing the largest zones of inhibition) corresponded to a peptide of mass 3348 (data not shown), with DNA sequence analysis confirming the presence of the expected serine to proline at position 29 (S29P) combined with an isoleucine to valine substitution at position 30 (I30V) in all cases.

The nisin A S29P-I30V (from here on termed nisin PV) derivative was selected for purification and specific activity assays. In contrast to the S29P variant, when nisin PV was purified by the standard RP-HPLC protocol (Supplementary Fig. 1B), it was found to be produced at near similar levels as the wild-type nisin A (data not shown). Moreover, the nisin PV peptide was stable during purification, lyophilisation and subsequent storage as determined by MS (data not shown). Given that valine is present at position 30 in the natural variants nisin F and nisin Q (Fig. 1), we carried out saturation mutagenesis at serine 29 in nisin Z, nisin F and also in nisin Q (Table S2). Mass spectrometry screening of the resultant transformant banks was used to identify 50 of the 60 potential derivatives of nisin Z, nisin F and nisin Q at the serine 29 location (Table S3) including the serine 29 to proline variants which were selected and purified for further analysis.

MIC-based investigations demonstrate enhanced specific activity of nisin A S29P, nisin PV, nisin F S29P, nisin Z S29P and nisin Q S29P.

To confirm that the enhanced activity of the variants nisin A S29P, nisin PV, nisin F S29P, nisin Z S29P and nisin Q S29P was due to increased specific activity, we assessed the activity of the purified peptides using classical broth-based MIC determination assays. In addition, a truncated nisin A^{1-28} peptide was generated and purified for comparative purposes. MIC assays were carried out using equimolar concentrations of parental nisin A and each nisin derivative against a range of bacterial targets, including the original *nsr*+ strain used in the screening process (*L. lactis* DRC3). This method established an MIC of 1.5 µg ml^{-1} (0.468 µM) for nisin A against *L. lactis* DRC3 (Table 1). In contrast, the MIC for nisin A S29P, nisin PV, nisin F S29P, and nisin Z S29P was 0.2 µg ml-1 (0.062 µM), equivalent to a 7.5-fold increase in specific activity compared to the wild type peptide. Moreover, Nisin Q S29P

exhibited a 15-fold increase in specific activity (0.1 μg ml^{-1}, 0.039 μM). The MIC of the truncated nisin A^{1-28} peptide (3 μg ml^{-1}, 0.937 μM) was 2-fold less than the value obtained for nisin A. To gain more insight into the specific activity of the engineered variants with respect to the presence or absence of NSR, we employed *L. lactis* MG1614 containing the plasmid pNP40 (*nsr*+) and its plasmid-free equivalent (*nsr*-) as indicator strains. In the case of *L. lactis* MG1614 pNP40, an MIC of 2 μg ml^{-1}(0.625 μM) was established for nisin A. In contrast, an MIC of 0.1 μg ml^{-1} (0.039 μM) was observed for nisin A, Z and F derivatives comprising the S29P substitution, reflecting a 16-fold increase in specific activity (Table 1). When assessed against the plasmid-free strain *L. lactis* MG1614, an MIC of 0.1 μg ml^{-1} (0.039 μM) was observed for nisin A, nisin A S29P, nisin PV, nisin F S29P, and nisin Z S29P (Table 1) and 0.05 μg ml^{-1} (0.019 μM) for nisin Q S29P.

Taken together, these results are in agreement with a previous study whereby NSR was shown to confer a 20-fold increase in resistance to nisin A in a *L. lactis* NZ9000 host strain when expressed from a plasmid (Khosa *et al.*, 2013). We also investigated the potency of the nisin variants against a panel of streptococci and enterococci, many strains of which have been shown to possess *nsr* genes (Khosa *et al.*, 2013), and three strains (*Streptococcus uberis* ATCC 700407, *S. uberis* DPC5344 and *Enterococcus casseliflavus* DPC 5053) were selected due to their extraordinary sensitivity to the S29P variants in agar diffusion assays (data not shown). A comparable MIC profile was observed for *S. uberis* ATCC 700407, *S. uberis* DPC5344 and *E. casseliflavus* DPC 5053 whereby an 8 to16-fold increase in MIC was observed for the S29P variant peptides compared to nisin A (Table 1). Subsequent whole genome sequencing of *S. uberis* ATCC 700407 and *S. uberis* DPC 5344 was carried out and the presence of *nsr* in each strain was confirmed (data not shown).

Growth curve-based comparisons of the activity of nisin A and nisin PV

Having ascertained the improved specific activity of nisin PV and the nisin F, Z and Q S29P variants against the representative *nsr*+ (*L. lactis* DRC3, *S. uberis* ATCC700407 and *S. uberis* 5344) strains through end-point MIC assays, further assessment was carried out by means of growth curves to investigate the impact of increasing concentrations of wild type peptide and serine to proline-based derivative peptides on bacterial growth (Fig. 3). We chose nisin PV as the representative bioengineered S29P derivative for comparative purposes with its parental and genetic background equivalent nisin A. In each instance, the results were consistent with the enhanced potency of nisin PV as revealed by MIC assays. Increasing concentrations of nisin PV resulted in an increasingly extended lag time, until such time that complete inhibition of bacterial growth was observed, highlighting its greater potency in comparison to the parental peptide against *L. lactis* DRC3 (Fig. 3A, 3B, 3C). Similar results were observed for *S. uberis* ATCC 700407 (Fig 3D, 3E, 3F) and *S. uberis* 5344 (Fig. 3G, 3H, 3I). Kill curve analysis also established the potency of nisin PV compared to nisin A against *L. lactis* DRC3 (Fig. S4).

Flow cytometric analysis to assess the viability of *L. lactis* DRC3 (NSR) in the presence of equimolar concentrations of nisin A or nisin PV

In order to compare the viability and vitality of bacterial cells in the presence of nisin A and nisin PV over a defined period of time, we assessed the effects of each peptide at a lethal concentration of 1.0 µg ml^{-1} (0.3 µM) against the *nsr*+ producing strain, *L. lactis* DRC3 (approximately 1 X 10^7 cfu ml^{-1}) in GM17 at 30 °C. As expected, a substantial increase in dead *L. lactis* DRC3 bacterial cells was observed after 60 mins incubation in the presence of nisin A and nisin PV, with nisin PV resulting in a statistically higher number of dead cells

than nisin A (P = 0.0002) (Fig. 4 and S3B). Accordingly, monitoring of live cells revealed decreases in cell numbers in samples treated with nisin A and nisin PV, with nisin PV eliciting a statistically greater reduction in live cells than nisin A (P = 0.0008) (Fig. 4 and S3A). Following incubation in GM17 broth at 30 °C for a further 60 mins, the number of dead cells triggered by nisin PV was statistically higher than corresponding numbers triggered by nisin A ($P = 8.52E^{-5}$) (Fig. 4 and S3B). Conversely, the number of live cells remaining after 120 mins of incubation was statistically lower when cells were treated with nisin PV compared to cells treated with nisin A (P = 0.001537) (Fig. 4 and S3A). Similar trends were noted when log-phase cells were treated with 0.3µM nisin A or nisin PV (Fig. S3)

Proteolytic activity of NSR against nisin A and nisin PV

The *in vivo* proteolytic activity of NSR was examined with a series of peptide release assays using nisin A and nisin PV as substrates in the presence of *L. lactis* DRC3 cells. Following incubation and centrifugation, nisin peptides or their degraded fragments in the supernatant were analyzed by RP-HPLC and mass spectrometry. For cells of *L. lactis* DRC3 incubated with nisin A, analysis revealed the absence of intact nisin (3354 Da) but detected the presence of one molecule with a smaller mass of 2,719.81 Da in the reaction buffer (Fig. 5A).

This corresponded to a nisin A peptide devoid of the C-terminal 6 amino acids ($nisin^{1–28}$) and verified that cleavage of the peptide bond between $MeLan^{28}$ and Ser^{29} had taken place. A fragment mass of 634 Da, corresponding to Ser^{29}-Lys^{34}, was not detected. For cells incubated with nisin PV, only intact peptide was detected (Fig. 5B), corresponding to the established mass of 3348 Da and reflecting the inability of NSR to cleave the peptide bond between $MeLan^{28}$ and Pro^{29} in this derivative.

Structural model of nisin and nisin PV bound to NSR

We carried out Molecular Dynamic (MD) simulations to predict the difference in molecular dynamics of the NSR protein in its interaction with the nisin C-terminus (residues 22-34) for nisin-A and nisin PV models. These simulations reveal the hydrogen bond occupancy (defined as whether a hydrogen bond is present or absent per picosecond over the duration of 50 nanoseconds) for all nisin residues greater than 20 % (Fig. 6C). It is evident that the nisin PV model exhibits higher occupancy values than the nisin A model (Fig. 6C) for residues corresponding to the region 27-29. In particular, there is a hydrogen bond with 47.65 % occupancy between the sidechain oxygen of proline in the nisin PV model and the hydrogen from the nitrogen sidechain on residue 265 (asparagine) in NSR that may play a role in the inhibition of proteolytic cleavage for nisin PV.

Analysis of the total binding energies for each residue in both nisin A and nisin PV models (Fig. 6A) reveals that at the critical residue 29, the total energy for proline is seven times greater than serine (4.6292kcal mol^{-1}/0.8775kcal mol^{-1} ~ 7). Indeed, the energies for nisin follow a particular pattern. Residues 22-25 (Lysine22 and ring D) are higher than those in the region corresponding to residues 27-29, whilst residues 30-34 are higher again. The findings of Khosa et al (Khosa *et al.*, 2016a) revealed a nisin A peptide that was bound more robustly for ring D-E and less so for residues 31-34. However our model is oriented in the opposite direction to that of Khosa et al (Khosa *et al.*, 2016a) such that residues 28-32 sit in the tunnel region of NSR. The tunnel is defined as the volume bounded by the active site region of the protease core (residues 168-174, 230-242) and the protease cap (residues 97-114, 262-285). Our MD simulations reveal that for wild type nisin, it is residues 30-32 that are most tightly bound. In contrast, for the model with proline at position 29, it is residues 29-32 that are the most firmly bound (Fig. 6A). The interesting observation here is that the residues within the tunnel region are those which are most strongly attached. In the study by

Khosa et al (Khosa *et al.*, 2016a) the residues that are most firmly attached (rings D+E and residue 30) are also observed to be in the tunnel region. This indicates that the NSR active site exhibits comparable binding behaviour regardless of the orientation of nisin.

Fig. 6B reveals how the root-mean-square deviation (RMSD) of the nisin residues changes over time. This is a measure of how these residues change structurally at each picosecond of the simulation. Each residue is compared to its position in the first picosecond and its RMSD plotted for every picosecond. Initially, both models quickly deviate from their reference structure by approximately 3 angstroms (this happens over the course of the first 0.3 nanoseconds during the heating, equilibration and the start of the production phase of the simulation). From then on in the simulation, the nisin A model continues to deviate until it reaches approximately 5 angstroms. In contrast, the nisin PV model exhibits a different behaviour. Although it too initially deviates further to almost 5 angstroms early in the simulation it then returns to just under 4 angstroms deviation from its reference structure and remains virtually constant for the rest of the simulation. This evidence of greater structural change in the nisin A model that is less apparent in the nisin PV model supports the findings of the binding energy analyses for both bond and total energies. Indeed, it is clear that the nisin PV model is more durably bound to the NSR protein and is certainly more robust than the nisin A model. Notably, the distance between the cleave point in the nisin molecule and the active site in NSR changes for both models (Fig. 6D). The most striking observation is that for the nisin PV model this distance remains relatively constant at approximately 6.25 angstroms thereby providing strong evidence of a rigidity and inflexibility in the region around the nisin cleave point. In contrast, the change in distance for the nisin A model fluctuates and displays an upward trend. For the purpose of the simulation, the cleave point was chosen to be at the nitrogen end of the peptide bond between serine 29 and cysteine.

Finally, a close up view of the bonds between residue 28 and residue 29 (proline) provides compelling evidence for the inability of NSR to cleave at residue 29 in the nisin PV model. Instead of the expected N-C peptide bond there appears to be a small network of 4 different bonds (Fig. 6F) joining cysteine to proline (CA-CD, C-CD, O-CD, O-CG) (C, CA and O are the atoms on the cysteine side of the bonds and CG and CD are the atoms on the proline side of the bonds where C is the alpha carbon and CA CD CG refer to the other carbons in relation to the alpha carbon within the amino acid). Of course this is how the molecular visualisation software interprets the positions of atoms in residue 28 relative to the positions of atoms in residue 29 (proline) and is reflective of the xyz coordinates listed in the pdb file for these atoms. According to the pdb file the positions of the atoms CA, C, and O in residue 28 relative to atoms CD and CG in residue 29 are extremely close, which in angstroms for each of the above bonds is 1.911, 0.743, 0.895, 1.239 respectively, thereby increasing substantially the magnitude of the intermolecular forces. Then in this region, the nisin PV model has enhanced robustness and the NSR protein will be restricted in its ability to move and cleave the antibiotic.

Discussion

Lantibiotics such as nisin possess many desirable characteristics for the treatment of infections caused by multi-drug resistant bacteria and consequently have attracted considerable interest as promising alternatives to existing antibiotics (Bierbaum & Sahl, 2009). Crucially, while antibiotic resistance has become a global issue, significant resistance to nisin outside of the laboratory has yet to be reported despite its widespread use as a food preservative (Breukink & de Kruijff, 1999). Recently however, several operons have been described that encode a protein defence mechanism against lantibiotics in strains that do not

produce such an antimicrobial (Khosa *et al.*, 2016b). Of considerable concern is the fact that the majority of these operons are found in human pathogenic strains of *Staphylococcus aureus, Listeria monocytogenes, Clostridium difficile, Bacillus cereus, Streptococcus mutans* and *Enterococcus faecium,* among others (Draper *et al.*, 2015). Such resistance mechanisms can involve (i) two-component systems (TCS) that are functionally and genetically linked to ABC transporters, (ii) the expression of functional immunity homologues such as those found in lantibiotic producing strains (e.g. NisI) (Alkhatib *et al.*, 2012) or (iii) the expression of dedicated enzymes that inactivate the lantibiotic peptide through chemical alteration (e.g. Nisinase)(Jarvis & Farr, 1971) or proteolytic cleavage (e.g. Nisin Resistance Protein) (Draper *et al.*, 2015). Furthermore, studies have indicated that some patterns of nisin resistance also play a part in the resistance to other antimicrobials or antibiotics (Zhou *et al.*, 2014). Consequently, novel inhibitors and alternative methods are required to counteract these systems which have the potential to effectively hamper the expanded use of lantibiotics as therapeutics.

Bioengineering (engineering within the cell) and the use of synthetic biology-based (*in vitro* engineering) approaches have been important for advancing our understanding of the fundamentals of bacteriocin activity and structure–function relationships and also in designing novel peptides with enhanced functionalities (activity and/or stability) which make them more attractive from a clinical perspective (Cotter *et al.*, 2013). Several examples of bioengineered variants of the prototypical lantibiotic nisin have been generated that provide examples of how lantibiotic functionality can be improved through single, or a small number of residue replacements ((Kuipers *et al.*, 1992, Healy *et al.*, 2013, Ge *et al.*, 2016, Li *et al.*, 2018, Zhou *et al.*, 2016).

Here, we screened a complete bank of nisin serine 29 derivatives and found just one, corresponding to a serine to proline change, which appeared to resist proteolytic cleavage by NSR. This observation is not unexpected given that proline is the only proteinogenic imino acid and is not accepted by many serine proteases including thermitase, subtilisin BPN' and proteinase K at the potential cleavage site (Brömme *et al.*, 1986). Indeed, substitution of amino acid residues by proline to increase proteolytic resistance is termed the 'proline concept' (Frenken *et al.*, 1993). Our molecular modelling studies effectively demonstrate an increased rigidity in the nisin PV model that most likely impedes the binding of the protease to the substrate. There appears to be a remarkable topology that provides an explanation for the observed differences in the effect of the proline mutation in countering the resistance of NSR. Out of all twenty amino acids it is only proline that causes the largest inhibition zone (Fig. 2). The topology of atoms in the region connecting residue 28 to residue 29 in nisin PV is such that NSR must overcome the binding energies resulting from the strength of the intermolecular forces derived from this topology. The closeness of each atom within this unique configuration provides a robustness to nisin that is rarely observed in other peptide bonds. Surprisingly, however, the S29P derivative retains significant bioactivity despite the structural changes associated with incorporation of proline residues, which can confer unique structural constraints on peptide chains (Yaron *et al.*, 1993), and in the case of nisin has often led to reduced specific activities (Field *et al.*, 2012, Field *et al.*, 2008). Follow-on MIC studies revealed the S29P peptides to be as active as nisin A against a variety of non-NSR expressing strains despite the restrictions of the peptidyl prolyl peptide bond, though some issues with peptide stability were apparent as indicated by increased susceptibility to oxidation. Consequently, a PCR-based bioengineering strategy specifically simultaneously targeting both serine 29 and isoleucine 30 of nisin A for mutagenesis was employed and ultimately led to the identification of nisin PV (proline and valine in place of serine 29 and

isoleucine 30, respectively). Importantly, this derivative was less vulnerable to oxidation during purification. Furthermore, an assessment of the specific activity of nisin PV against *L. lactis* MG1614 pNP40 (*nsr*+) revealed a 20-fold increase when compared to the wild type peptide. Indeed, at either sub-inhibitory or lethal levels, the nisin PV derivative consistently outperformed nisin A and appeared to elicit a faster killing effect on the NSR producer, *L. lactis* DRC3. Peptide release assays were able to confirm the inability of NSR to proteolytically cleave at the proline peptide bond, thereby negating the benefits of NSR by restoring the specific activity to levels observed against the same strain lacking the enzyme.

This study emphasizes the benefits of random mutagenesis in combination with rational design approaches to peptide engineering and also establishes for the first time that stable nisin derivatives can be created that possess enhanced resistance to proteolytic destruction, whilst retaining full potency and spectrum of antimicrobial activity against bacteria of clinical significance. However, beneficial substitutions can often produce other unanticipated impacts on peptide functionality, as was observed by nisin A S29P and nisin PV that exhibited a slightly reduced induction capacity compared to parental nisin A when assessed by a GFP reporter system (Figure S6A).

Notably, *nsr* genes are frequently located on naturally *transmissible* elements (Froseth & McKay, 1991, Liu *et al.*, 1997, Tang *et al.*, 2001), underlining the potential ease of transference of NSR-mediated resistance to other bacteria. Significantly, many of the strains shown to possess *nsr* genes include streptococci of bovine origin (Khosa *et al.*, 2013), such as *S. agalactiae* ATCC13813 (bovine isolate UK), *S. dysgalactiae* susbp. *dysgalactiae* ATCC 27957 (isolated from a bovine udder infection and used as a control strain for mastitis screening), and *Streptococcus canis* FSL Z3-227 (milk isolate from a cow with an intra-mammary infection) (Richards *et al.*, 2012). From a human medicine perspective, streptococci are commonly found in the human gastrointestinal, reproductive and urinary

tracts and can cause life-threatening diseases in neonates, pregnant women, the elderly, and adults with compromised immune systems. They have attracted recent attention because of newly emerging, antibiotic-resistant strains (Dogan *et al.*, 2005, Da Cunha *et al.*, 2014). NSR-like genes have also been uncovered in human isolates including *S. agalactiae* COH1 (isolated from a case of fatal infant septicaemia belonging to the hypervirulent ST-17 lineage) (Tettelin *et al.*, 2005), *S. agalactiae* 2603 V/R (human blood isolate) and *S. dysgalactiae* subsp. *equisimilis* AC-2713 (an isolate from a human blood infection) (Brandt *et al.*, 1999). Moreover, comparative sequence analysis using the NSR protein from *L. lactis* DRC3 as the query sequence (Fig. S5) revealed the presence of homologues in other animal and human pathogens including *Streptoccocus suis* (a zoonotic porcine opportunistic pathogen), *S. canis* (an emerging bovine and human pathogen (Douglas *et al.*, 1993)), *Streptococcus gordonii* (most frequently identified as being primary aetiological agent of subacute bacterial endocarditis), *Staphylococcus hyicus*, *Staphylococcus epidermidis* and *E. faecium* (long recognized as important human pathogens).

Since their discovery, antibiotics have been a reliable means for treating many infections. However, antibiotics have driven the expansion of resistant organisms making once routine infections significantly more difficult to treat. The situation is exacerbated by the lack of progress with respect to the clinical development of new antibiotics. It has frequently been suggested that nisin could have tremendous value in clinical settings as a result of its high potency *in vitro*, duality in mechanistic action and the capacity to kill target cells rapidly (Cavera *et al.*, 2015). However, if nisin is to be deployed as an effective therapeutic in the fight against multi-drug resistant pathogens, the implementation of strategies to prevent or curtail resistance development to nisin and currently available antibiotics in the future is paramount. One such option could involve different nisin variants (such as the derivatives described in this study) in combination with conventional drugs to

promote synergistic outcomes. Treatment with a combination of nisin and a traditional antibiotic would theoretically reduce the incidence of resistance. Indeed, several studies have demonstrated synergistic relationships between conventional antibiotics and nisin against bacteria such as staphylococci, including methicillin-resistant forms (Piper *et al.*, 2009, Dosler & Gerceker, 2011, Okuda *et al.*, 2013), enterococci (Tong *et al.*, 2014), including vancomycin-resistant enterococci (Brumfitt *et al.*, 2002), and streptococci (Lebel *et al.*, 2013), *Pseudomonas* (Naghmouchi *et al.*, 2012, Naghmouchi *et al.*, 2013) as well as *E. coli* and *Klebsiella* (Field *et al.*, 2016). These studies highlight the merits of employing antibiotic combination strategies to enhance the efficacy of available antibiotics, and ultimately, reduce the potential for resistance issues to arise in clinical settings.

Ultimately, a comprehensive understanding of NSR and NSR-like proteolytic resistance mechanisms will be invaluable, providing strategic information for further tailoring of genetic variants of lantibiotic peptides that circumvent protein defence systems. Indeed, if nisin is to further expand its applications in the biomedical field, it will be critical to examine and monitor nisin resistance development in pathogenic organisms in preparation for any potential resistance issues that may arise in the future. In addition, more extensive investigations should be undertaken to evaluate not only the prevalence of *nsr*+ strains in nature, but also the potential for the transfer of *nsr* genes from non-pathogenic organisms to other clinically relevant pathogenic microbes. Such knowledge may also aid in the development of appropriate therapeutic regimens to overcome such issues should nisin be deployed in clinical settings. The production of nisin peptides with value-added properties such as the nisin A S29P, nisin PV, nisin F S29P, nisin Z S29P and nisin Q S29P derivatives described in this study reinforces our belief that bioengineering strategies can be successfully employed to overcome the many challenges associated with peptide antimicrobials including antimicrobial activity, heat stability, solubility, diffusion, and as described here, protease

sensitivity. Critically, the need for robust and economically feasible industrial scale production processes will be an essential requirement for further clinical development, in addition to thorough assessment in clinical trials to determine or substantiate *in vivo* efficacy.

Experimental Procedures

Bacterial Strains and Growth Conditions

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

Nisin A S29XX derivatives (in which Serine 29 and Isoleucine 30 are randomized together).

Saturation mutagenesis of the serine and isoleucine codons at position 29 and 30 respectively of *nis*A was carried out with pDF05 (pCI372-nisA) as template and using oligonucleotides **NisA**S29XXdeg FOR and **NisA**S29XXdeg REV (see Table S2 in the Supplementary material) containing an NNK codon in place of each native codon. The purified products were subsequently introduced by electroporation into the strain NZ9800 which has all the genes necessary for Nisin production. Approximately 2000 transformants were chosen at

random using a Genetix QPIX II-XT colony-picking robot and added to GM17 Cm^{10} within 96-well plates, incubated overnight at 30 ^{o}C and stored at -80 ^{o}C.

Nisin Z, Nisin F and Nisin Q S29X derivatives

Saturation mutagenesis of the serine codon at position 29 of *nis*Z was carried out with pDF05 (pCI372-nisA) as template and using oligonucleotides **NisZ**S29deg FOR and **NisZ**S29deg REV (Supplementary Table 2) containing an NNK codon in place of each native codon (codons to alter His27Asn are underlined). Saturation mutagenesis of the serine codon at position 29 of *nis*Z was carried out with pDF05 (pCI372-nisA) as template and using oligonucleotides **NisF**S29deg FOR and **NisF**S29deg REV containing an NNK codon in place of each native codon (codon changes for H27N and I30V are underlined). Saturation mutagenesis of the serine codon at position 29 of *nis*Q was carried out with pCI372-*nis*Q as template and using oligonucleotides **NisF**S29deg FOR and **NisQ**S29deg REV containing an NNK codon in place of each native codon (codon changes for H27N and I30V and M21L are underlined). The purified products were subsequently introduced by electroporation into the strain *L. lactis* NZ9800 which has all the genes necessary for nisin production. Approximately 180 transformants were chosen at random from each nisin background and inoculated into 96 well plates containing GM17 Cm^{10}, incubated overnight and stored at -20 ˚C after addition of 80 % glycerol.

Creation of a truncated nisin A$^{1-28}$ derivative

Mutagenesis of the *nis*A gene was carried out as described previously (Field *et al.*, 2008). Briefly, saturation mutagenesis was carried out using pDF05 (pCI372-*nisA*) as template and using oligonucleotides NisA1-28 For and NisA1-28 Rev as listed in (see Table S2 in the supplemental material) containing a TAA stop codon in place of the native AGT codon (serine 29). PCR amplification was performed in a 50 µl reaction containing approximately 0.5 ng of target DNA (pDF05), 1 unit Phusion High-Fidelity DNA polymerase (Finnzymes, Finland), 1 mM dNTPs and 500 ng each of the appropriate forward and reverse oligonucleotide. The reaction was pre-heated at 98 °C for 2 min, and then incubated for 29 cycles at 98 °C for 30 s, 55 °C for 15 s and 72 °C for 3 min 30 s, and then finished by incubating at 72 °C for 3 min 30 s. Amplified products were treated with Dpn1 (Stratagene) for 60 min at 37 °C to digest template DNA and purified using the QIAquick PCR purification kit. Following transformation of *E. coli* Top 10 cells (Invitrogen, Dublin, Ireland), plasmid DNA was isolated and sequenced using primers pCI372FOR and pCI372REV (Table S1) to verify that mutagenesis had taken place. The purified products were subsequently introduced by electroporation into the strain *L. lactis* NZ9800.

Bioassays for antimicrobial activity

Deferred antagonism assays were performed by replicating strains on GM17 agar plates and allowing them to grow overnight before overlaying with either GM17 agar (0.75 % w/v agar) or seeded with the nisin resistance protein-expressing indicator strain *L. lactis* subsp. *lactis* biovar diacetylactis DRC3 or *Streptococcus uberis* respectively. For higher throughput screening of the S29XX bank, deferred antagonism assays were performed by replicating strains using a 96 pin replicator (Boekel) or spotting 5 µl of a fresh overnight culture on

GM17 agar plates and allowing them to grow overnight. Following overnight growth, the strains were subjected to UV radiation for 30 minutes prior to overlaying with either GM17 or BHI agar (0.75 % w/v agar) seeded with the appropriate indicator.

Nisin purification

L. lactis NZ9700 (nisin A producer) or the mutant nisin strain of interest was sub-cultured twice in GM17 broth at 1 % at 30°C before use. Two litres of modified Tryptone Yeast broth were inoculated with the culture at 0.5 % and incubated at 30°C overnight. The culture was centrifuged at 7,000 rpm for 15 minutes. The cell pellet was resuspended in 300 ml of 70 % isopropanol (IPA) 0.1 % trifluoroacetic acid (TFA) and stirred at room temperature for approximately 3h. The cell debris was removed by centrifugation at 6250 g for 15 minutes and the supernatant retained. The isopropanol was evaporated using a rotary evaporator (Buchi) and the sample pH adjusted to 4 before applying to a 10 g (60 ml) Varian C-18 Bond Elution Column (Varian, Harbor City, CA) pre-equilibrated with methanol and water. The columns were washed with 100 mls of 20 % ethanol and the inhibitory activity was eluted in 100 mls of 70 % IPA 0.1 % TFA. 15 ml aliquots were concentrated to 2 ml through the removal of propan-2-ol by rotary evaporation. 1.5 ml aliquots were applied to a Phenomenex (Phenomenex, Cheshire, UK) C12 reverse phase (RP)-HPLC column (Jupiter 4u proteo 90 Å, 250 × 10.0 mm, 4 µm) previously equilibrated with 25 % propan-2-ol, 0.1 % TFA. The column was subsequently developed in a gradient of 30 % propan-2-ol containing 0.1 % TFA to 60 % propan-2-ol containing 0.1 % TFA from 10 to 45 minutes at a flow rate of 1.2 ml min^{-1}.

Mass Spectrometry

For Colony Mass Spectrometry (CMS) bacterial colonies were collected with sterile plastic loops and mixed with 50 µl of 70 % IPA adjusted to pH 2 with HCl. The suspension was vortexed, the cells centrifuged in a benchtop centrifuge at 8260*g* for 2 mins, and the supernatant was removed for analysis. Mass Spectrometry in all cases was performed with an Axima CFR plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10 mg ml^{-1} in 50 % acetonitrile-0.1 % (v/v) TFA) was placed onto the target and left for 1-2 mins before being removed. The residual solution was then air-dried and the sample solution (resuspended lyophilised powder or CMS supernatant) was positioned onto the precoated sample spot. Matrix solution (0.5 µl) was added to the sample and allowed to air-dry. The sample was subsequently analysed in positive-ion reflectron mode.

Minimum Inhibitory Concentration assays

Minimum inhibitory concentration (MIC) determinations were carried out in triplicate in 96 well microtitre plates. 96 well microtitre plates were pre-treated with bovine serum albumin (BSA) prior to addition of the peptides. Briefly, to each well of the microtitre plate 200 µL of phosphate buffered saline (PBS) containing 1 % (w/v) bovine serum albumin (PBS/BSA) was added and incubated at 37 °C for 30 min. The wells were washed with 200 µL PBS and allowed to dry. Target strains were grown overnight in the appropriate conditions and medium, subcultured into fresh broth and allowed to grow to an OD_{600} of ~0.5, diluted to a final concentration of 10^5 cfu ml^{-1} in a volume of 0.2 ml. Wild type nisin and nisin mutant peptides were adjusted to a 20 µM or 5 µM (streptococci), 7.5 µM or 500 nM (*L. lactis*) and 10 µM (enterococci) starting concentration and 2-fold serial dilutions of each peptide were

added to the target strain. After incubation for 16 h at 37 °C the MIC was read as the lowest peptide concentration causing inhibition of visible growth.

Growth curve-based comparisons of the activity of nisin A and nisin PV

For growth experiments, overnight cultures were transferred (10^7 cfu ml^{-1} in a volume of 1.0 ml.) into GM17 (*Lactococcus*) or TSB-YE (*Streptococcus*) supplemented with the relevant concentration of wild-type and PV peptides, and subsequently 0.2 ml was transferred to 96 well microtitre plates (Sarstedt). Cells were grown statically at 30 °C for lactococci and 37 °C for streptococci and measured spectrophotometrically over 24-h periods at 1 hour intervals (with shaking for 5 seconds before each reading) using a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, California, USA). Experiments were carried out in triplicate.

Assays for nisin cleavage activity of NSR against nisin A and nisin PV

To investigate whether NSR could degrade the nisin A mutant PV *in vivo*, a series of peptide release assays were performed according to the method described by Stein et al (Stein *et al.*, 2003). Briefly, flasks containing 50 ml of GM17 medium were inoculated with a 1/100 volume of overnight of *L. lactis* subsp. *lactis* biovar diacetylactis DRC3 and incubated at 30 °C until the OD_{600} reached 0.6. Cells were harvested by centrifugation at 4,000 *g* for 10 min, washed twice with 50 mM Tris-HCl (pH 6.0), centrifuged again, and resuspended in incubation buffer (50 mM sodium phosphate buffer [pH 6.0], 1 M NaCl, and 1 % [wt/vol] glucose) at 1 ml aliquots in microcentrifuge tubes. Nisin A and nisin PV (50 μg) was added, and the aliquots were incubated for 30 min at 30 °C with gentle shaking. After incubation, the

aliquots were centrifuged for 10 min at full speed in a microcentrifuge, and the supernatants collected. The harvested cell pellets were washed with the incubation buffer, gently mixed with 1 ml of 20 % acetonitrile in water containing 0.1 % TFA, and incubated with gentle shaking at 30 °C for 5 min. The cells were removed by centrifugation at 12,000 *g* for 10 min, and the supernatants were collected. The collected supernatants (500 µl each) were subjected to RP-HPLC analysis as described above.

Evaluation of cell viability using flow cytometry

L. lactis DRC3 was grown overnight for 16 hours in GM17 broth at 30 °C under static conditions. From overnight cultures, 100 µl samples were centrifuged at 8260*g* for 10 minutes and resuspended in 1 ml of fresh GM17 broth. After thoroughly mixing and vortexing, 100 µl of this resuspended mixture was added to 900 µl of 0.3 µM nisin A, compared to 0.3 µM nisin PV (peptides were also resuspended in fresh GM17 broth). Thus, the overnight samples were diluted 10^{-2} to obtain a starting concentration of approximately 10^7 cfu ml^{-1}. Log-phase DRC3 cells were also assessed by subculturing overnight cultures at 4 % for 4 hrs in GM17 broth at 30 °C. One hundred microliter aliquots of log-phase cells were added to 900 µl of 0.3 µM nisin A versus 0.3 µM nisin PV, such that the starting concentration of log-phase cells was also approximately 10^7 cfu ml^{-1}. At every time point, 75 µl aliquots were taken from the nisin-treated samples and further diluted 1/10 in phosphate buffered saline (PBS), prior to staining with the cell viability probes Syto9 and propidium iodide (PI) (Live/Dead® BacLight™ viability kit (Molecular Probes, Eugene, Oregon, USA). 150 µl of these diluted samples were stained, such that the final concentrations of Syto9 and PI were 6.68 µM and 40 µM respectively. Samples were stained for 10 minutes in the dark at 30 °C, prior to analysis by flow cytometry (BD Accuri C6). Analysis of stained samples was performed for 1 min at medium flow rate (35 µl/sec) with a BD Accuri C6 flow cytometer

(Beckton Dickinson, Belgium). A threshold setting of 10000 on FSC was utilised in order to minimize background. Acquisition and analysis of Syto 9 (FL1) and PI fluorescence (FL3, > 670 nm long pass) was conducted using BD Accuri C6 software v. 1.0.2 (Beckton Dickinson, Belgium). Samples were taken at time points 0 min, 20 min and 40 min (for log-phase samples treated with 0.3 µM nisin peptides) and time points 0 min, 60 min and 120 min (for overnight samples treated with 0.3 µM nisin peptides), for analysis by flow cytometry. Negative controls included approximately 10^7 cfu ml^{-1} *L. lactis* DRC3 samples resuspended in GM17 broth in the absence of nisin in all cases. Healthy cells without any membrane damage exhibit green fluorescence due to cellular uptake of Syto9. In contrast, dead cells with damaged membranes stain red due to PI. Stressed/injured cells contain a permeabilized membrane resulting in the uptake of both dyes in a ratio dependent on the extent of membrane damage and thus exhibit orange fluorescence. A total of 9 replicates were utilised for flow cytometry experiments with overnight cells and a total of 6 replicates were utilised for experiments with log-phase cells.

Structural model of nisin and nisin PV bound to NSR

Two molecular models were used to investigate the difference in the simulated interaction between NSR and nisin A and the interaction between NSR and nisin PV. The initial coordinates for the nisin A model were taken from the 1wco protein databank file (Hsu *et al.*, 2004). The AutodockTools program (Morris *et al.*, 2009) was then used to determine the starting configuration for the serine model interacting with NSR. Using the starting configuration of the nisin A model, the CHIMERA program was next used to mutate serine 29 and isoleucine 30 to proline 29 and valine 30 respectively which was the saved as the nisin PV model pdb file (Pettersen *et al.*, 2004). Then Autodock was used to determine the most favorable binding configuration to NSR for the PV model as the starting configuration for the

nisin PV simulation. The full procedure for creating the starting files can be found in the supplementary methods along with the procedure for charge neutralising, system solvating, minimising, thermalising and equilibrating in both NSR-nisin systems.

Statistical analysis:

Flow cytometry data was analysed using a 1-tailed t-test to show that there was a statistically significant difference in the sub-populations for samples treated with nisin PV compared to samples treated with nisin A. The difference in this case being that the mean live cell count of the sub-population treated with nisin A was greater than the mean live cell count of the sub-population treated with nisin PV. This would then be statistical evidence at the 5 % level of significance that nisin PV is more resistant to NSR than nisin A as there are more nisin A treated cells with "live" bacteria than there are with nisin PV treated cells. To account for this statistically, we state that the Null hypothesis is H_0: $\mu_1^{Live} - \mu_2^{Live} = 0$ where μ_1^{Live} is the mean live cell count of the sub-population treated with nisin A and μ_2^{Live} is the mean live cell count of the sub-popuation treated with nisin PV. Then the alternative hypothesis is H_1 : $\mu_1^{Live} - \mu_2^{Live} > 0$. Conversely, in order to anlayse the dead cell population, the null hypothesis is that H_0: $\mu_2^{Dead} - \mu_1^{Dead} = 0$ and the alternative hypothesis is H_1: $\mu_2^{Dead} - \mu_1^{Dead} > 0$.

Kill curve statistical analysis was carried out using an unpaired Students t-test in GraphPad Prism software following analysis by SPSS to show data was normally distributed.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon request. All data and software code including a detailed workflow for the Molecular Dynamics Simulations can be accessed at the following github repository. https://github.com/tony-blake/MD-Simulation

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Author contributions

DF, PDC and CH conceived the study. DF constructed and analysed the nisin mutant banks using MIC, kill and growth curve analysis and peptide release assays. HM carried out flow cytometry experiments and analysed the data. TB carried out molecular dynamics simulations and analysis. POC carried out Mass Spectrometry. D.F., H.M., and T.B drafted the initial manuscript. P.D.C., C.H and R.P.R. critically revised the drafted manuscript. All authors read and approved the final version of the manuscript prior to submission.

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Table 1. Specific activity of nisin A S29P, nisin PV, nisin F S29P, nisin Z S29P and nisin Q S29P against a range of NSR-expressing and non-expressing indicator organisms. Bold font denotes enhanced specific activity compared to wild type nisin A. ND = not determined.

Indicator organism	**Nisin A WT** **µg ml^{-1} (µM)**	**Nisin A PV** **µg ml^{-1} (µM)**	**Nisin A S29P** **µg ml^{-1} (µM)**	**Nisin F S29P** **µg ml^{-1} (µM)**	**Nisin Z S29P** **µg ml^{-1} (µM)**	**Nisin Q S29P** **µg ml^{-1} (µM)**	**Nisin A 1-28** **µg ml^{-1} (µM)**
L. lactis subsp. diacetylactis DRC3	1.5 (0.468)	**0.2 (0.062)**	**0.2 (0.062)**	**0.2 (0.062)**	**0.2 (0.062)**	**0.1 (0.039)**	3 (0.937)
L. lactis MG1614	0.1 (0.039)	0.1 (0.039)	0.1 (0.039)	0.1 (0.039)	0.1 (0.039)	**0.05 (0.019)**	1.25 (0.375)
L. lactis MG1614 pNP40	2(0.625)	**0.1 (0.039)**	**0.1 (0.039)**	**0.05 (0.019)**	**0.05 (0.019)**	**0.05 (0.019)**	3 (0.937)
S. uberis ATCC 700407	16 (5)	**2 (0.625)**	**2 (0.625)**	**1 (0.312)**	**1 (0.312)**	**1 (0.312)**	48 (15)
S. uberis DPC 5344	16 (5)	**4 (1.25)**	**4 (1.25)**	**2 (0.625)**	**2 (0.625)**	**2 (0.625)**	48 (15)
E. casseliflavus DPC 5053	8(2.5)	**1 (0.312)**	**1 (0.312)**	**1 (0.312)**	**1 (0.312)**	**0.5 (0.156)**	25 (7.5)

Figure 1. Structure of nisin A. Residues are represented in the single letter code. Post translational modifications are indicated as follows, Dha: dehydroalanine, Dhb: dehydrobutyrine, Abu: 2-aminobutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: 3-methyllanthionine. Orange circles indicate amino acid differences between the natural nisin variants Z, F and Q. The Nisin Resistance Protein (NSR) cut-site is indicated by red arrow.

Fig. 2 Deferred antagonism assay of nisin A serine 29 derivatives against *Lactococcus lactis* subsp diacetylactis DRC3 (NSR+). Zones of inhibition produced by a bank of nisin A derivatives which have been randomized at Serine 29 against the indicator *Lactococcus lactis* subsp diacetylactis DRC3 which expresses the nisin resistance protein (NSR) from the plasmid pNP40. Single letters correspond to IUPAC abbreviation code, wt=Serine.

Fig. 3 Impact of increasing concentration of nisin A and nisin PV on growth. Effect of nisin A and nisin PV on *L. lactis* subsp diacetylactis DRC3 nisin A (green square), nisin PV (red triangle) and untreated control (blue circle) in 0.16 µg ml^{-1} (A), 0.32 µg ml^{-1} (B) and 0.64 µg ml^{-1} (C), *S. uberis* ATCC 700407 in 0.96 µg ml^{-1} (D), 1.28 µg ml^{-1} (E) and 1.92 µg ml^{-1} (F), and *S. uberis* DPC5344 in 6.4 µg ml^{-1} (G), 9.6 µg ml^{-1} (H) and 12.8 µg ml^{-1} (I). The means and standard deviations of three independent determinations are presented.

Fig. 4 Flow cytometry analysis and representative plots of *L. lactis* subsp *lactis* biovar diacetylactis DRC3 cells (1 X 10^7) treated with 1.0 µg ml^{-1} (0.3µM) nisin A, nisin PV or GM17 media as a negative control, indicating percentages of live, injured, dead and fragmented cells, employing a quadrant-based gating strategy. Populations of live (Syto9) and dead (PI) cells are visible on a PI (FL3-H) vs Syto9 (FL1-H) plot. Samples were analysed upon addition of peptides (T0) and subsequently at 60 mins (T60) and 120 mins (T120).

Fig. 5 Peptide release assay of nisin A and nisin PV in the presence of NSR reveals truncated nisin A but intact nisin PV. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) analysis of (**A**) purified nisin A peptide following incubation with *L. lactis* subsp diacetylactis DRC3 (nsr+). Asterisk denotes fraction corresponding to the digested and truncated nisin A^{1-28} fragment as determined by mass spectrometry (inset) and (**B**) purified nisin PV following incubation with *L. lactis* subsp diacetylactis DRC3 (nsr+). Asterisk denotes fraction corresponding to intact nisin PV as determined by mass spectrometry (inset).

Fig. 6 MD simulations of NSR-nisin A and NSR-nisin PV model complexes. (A) Mean net binding energy per residue in nisin A and nisin PV over the duration of 50 ns. For residue S29P, proline has a much greater binding energy than serine. (B) Backbone RMSD for Nisin A (red) and Nisin PV (green) over a trajectory of 50 ns. (C) All hydrogen bond occupancies over a trajectory of 50 ns that are greater than 0.1. (D) Change in distance between cleavage point at nitrogen in Residue 28 in nisin and the Sidechain Oxygen in SER236 in NSR over 50 ns. Lines were smoothed using ggplot2's geom smooth function. (E) Comparison view of nisin A and nisin PV inside tunnel region of NSR at start of MD simulation. (F) A network of bonds is evident in nisin PV at cleave point location.