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Studies with bioengineered Nisin peptides highlight the broad-spectrum potency of Nisin V

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Summary

Nisin A is the most thoroughly investigated member of the lantibiotic family of antimicrobial peptides. In addition to a long history of safe use as a food antimicrobial, its activity against multi-drug resistant pathogens has resulted in a renewed interest in applying nisin as a chemotherapeutic to treat bacterial infections. The wealth of Nisin-related information that has been generated has also led to the development of the biotechnological capacity to engineer novel Nisin variants with a view to improving the function and physicochemical properties of this already potent peptide. However, the identification of bioengineered Nisin derivatives with enhanced antimicrobial activity against Gram-positive targets is a recent event. In this study, we created stable producers of the most promising derivatives of Nisin A generated to date [M21V (hereafter Nisin V) and K22T (hereafter Nisin T)] and assessed their potency against a range of drug-resistant clinical, veterinary and food pathogens. Nisin T exhibited increased activity against all veterinary isolates, including streptococci and staphylococci, and against a number of multi-drug resistant clinical isolates including MRSA, but not vancomycin-resistant enterococci. In contrast, Nisin V displayed increased potency against all targets tested including hVISA strains and the hyper-virulent *Clostridium difficile* ribotype 027 and against important food pathogens such as *Listeria monocytogenes* and *Bacillus cereus*. Significantly, this enhanced activity was validated in a model food system against *L. monocytogenes*. We conclude that Nisin V possesses significant potential as a novel preservative or chemotherapeutic compound.

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Introduction

The lantibiotics are an ever-expanding family of antimicrobial peptides that are produced by a diverse range of bacteria (McAuliffe *et al*., 2001; Twomey *et al*., 2002; Chatterjee *et al*., 2005; Bierbaum and Sahl, 2009). These gene-encoded, ribosomally synthesized peptides are distinguished by the presence of post-translationally modified amino acids such as dehydroalanine (Dha), dehydrobutyrine (Dhb) and the eponymous lanthionine (Lan) and β-methyllanthionine (MeLan) formed by thioether linkages between dehydrated residues and neighbouring cysteines. As a result of their highly potent biological activities, lantibiotics have the potential to be employed as novel antimicrobials to combat medically significant bacteria and their multi-drug resistant forms (Cotter *et al*., 2005a; Lawton *et al*., 2007; Piper *et al*., 2009a,b). The prototypical and most thoroughly investigated lantibiotic is Nisin A, a 34 amino acid polycyclic peptide that exhibits antibacterial activity against a wide range of clinical and food-borne pathogens, including staphylococci, bacilli, clostridia and *Listeria*. 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; Wiedemann *et al*., 2001; Bonelli *et al*., 2006). As a consequence of these two distinct and cooperative mechanisms, microbes have been unable to develop any significant resistance to Nisin A despite its widespread use in the food industry (Breukink and de Kruijff, 1999). Nisin A has a long record of safe use and has been approved as a natural biopreservative by the US FDA (United States Food and Drug Administration), the WHO (World Health Organization, 1969) and by the EU as additive E234 (Delves-Broughton, 1990; Chen and Hoover, 2003; Guinane *et al*., 2005) for use in a wide variety of foods including processed cheese, dairy products and canned foods (for reviews see Chen and Hoover, 2003; Deegan *et al*., 2006; Sobrino-Lopez and Martin-Belloso, 2008). Furthermore, the efficacy of both Nisin A and its natural variant Nisin Z against the Gram-positive pathogens responsible for bovine mastitis has resulted in

its incorporation into a number of products dedicated to controlling or treating such infections (Sears *et al*., 1992; Cotter *et al*., 2005a; Cao *et al*., 2007; Wu *et al*., 2007). The *in vivo* effectiveness of Nisin A has been tested and it was shown to be more effective than vancomycin when treating mice infected with *Streptococcus pneumoniae* (Goldstein *et al*., 1998) while multi-drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), heterogeneous vancomycin-intermediate *S. aureus* (hVISA) and vancomycin-resistant enterococci (VRE) have all been shown to be susceptible to Nisin A (Severina *et al*., 1998; Brumfitt *et al*., 2002; Piper *et al*., 2009b). Nisin F, another natural variant, was also found to successfully control *S. aureus* infection in rats (De Kwaadsteniet *et al*., 2009). Nisin A also effectively inhibits the microorganisms responsible for periodontal disease (Howell *et al*., 1993; Turner *et al*., 2004). Other applications have been suggested for Nisin A arising from its strong spermicidal (Aranha *et al*., 2004; Reddy *et al*., 2004; Gupta *et al*., 2009) and anti-fungal properties (Akerey *et al*., 2009).

It has been suggested that, due to its gene-encoded nature, the efficacy of Nisin A as an antimicrobial could be further improved through bioengineering-based approaches. Although such efforts were first made in the early 1990s, the results were disappointing in that, while bioengineered derivatives were generated that facilitated the elucidation of structure-function analyses or resulted in increased activity against Gram-negative or non-pathogenic targets (Kuipers *et al*., 1996; Yuan *et al*., 2004; Rink *et al*., 2007), no Nisin A or Z derivatives with enhanced potency against Gram-positive pathogens had been identified. The recent identification, following the utilization of random and site saturation mutagenesis approaches, of a number of Nisin A derivatives with enhanced bioactivity against Gram-positive pathogens was thus a significant development (Field *et al*., 2008). Specific activity studies with three of these Nisin A derivatives (M21V, K22T and N20P) revealed that M21V possesses enhanced specific activity against *S. aureus* DPC 5245 and *Listeria monocytogenes* EGDe and 10403S, that K22T possesses enhanced specific activity against *Streptococcus agalactiae* ATCC 13813 and *S. aureus* ST528 (MRSA) and that N20P possesses enhanced specific activity against *S. aureus* ST528 but reduced activity against *S. agalactiae* 13813 (Field *et al*., 2008). Here we create stable producers, and carry out a more detailed analysis, of the two bioengineered Nisin derivatives with enhanced broad spectrum activity relative to Nisin A [M21V (hereafter Nisin V) and K22T (hereafter Nisin T)]. This analysis incorporates a wider selection of strains and species of medically significant pathogens, including hVISA, VRE and additional MRSA strains, *Clostridium difficile* ribotype 027, *S. agalactiae* and *L. monocytogenes* isolates, including a mutant that exhibits enhanced Nisin A resistance. Notably, we demonstrate that the enhanced specific activity of Nisin V over Nisin A against *L. monocytogenes* F2365 is retained in food matrices.

Results

Creation of stable Nisin V- and Nisin T-producers

To facilitate ongoing Nisin V- and T-related research, we created producers, which are likely to be more genetically stable, through double cross-over homologous recombination. This yields strains which are useful for peptide purification, but also is a strategy which is less likely to impinge on the food-grade status of the producers as no heterologous DNA is present in the final constructs. The *nisV* and *nisT* genes (generated by PCR-based mutagenesis) were each inserted at the appropriate location in the *Lactococcus lactis* NZ9800 chromosome *via* double cross-over recombination to generate *L. lactis* NZ9800::*nis*V and *L. lactis* NZ9800::*nis*T. *Lactococcus lactis* NZ9800 is a derivative of NZ9700 that has a four-base pair deletion in the *nis*A gene (Kuipers *et al*., 1993). Results of deferred antagonism assays with *S. agalactiae* ATCC 13813 *and L. monocytogenes* EGDe indicated that gene replacement had successfully occurred and that the bioactivity profiles of the newly constructed strains differed from that of *L. lactis* NZ9700 (Fig. 1A and B). Mass

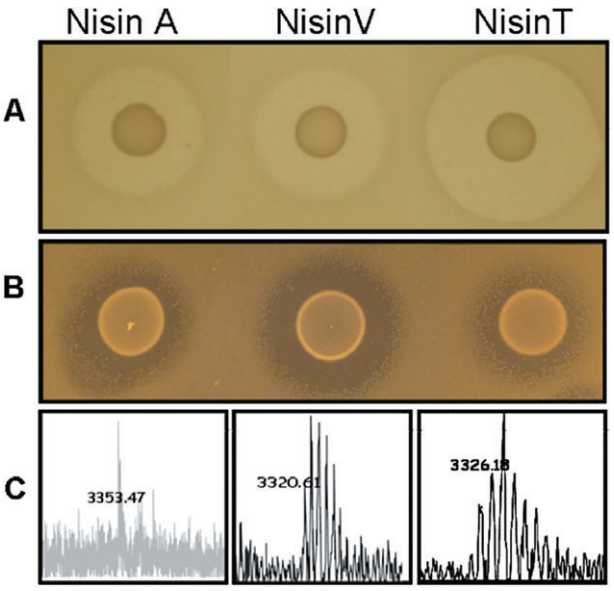

Fig. 1. A. Deferred antagonism assays of the Nisin A producing strain *L. lactis* NZ9700 and the stable nisin derivative producing strains *L. lactis* NZ9800::*nis*V and *L. lactis* NZ9800::*nis*T against the sensitive indicator *S. agalactiae* ATCC 13813.
B. *L. monocytogenes* EGDe.
C. Colony Mass Spectrometry analysis of the nisin A (3353 amu), Nisin V (3321 amu) and Nisin T (3326 amu) producing strains utilized in this study.

spectrometry analysis confirmed the production of peptides with masses corresponding to Nisin V (3321 amu) and Nisin T (3326 amu) (Fig. 1C). We also confirmed the absence of the pORI280 shuttle vector employed to facilitate the recombination process (data not shown). It was noted that these newly created Nisin V and T producers produced levels of peptide that corresponded closely with those produced when the corresponding structural genes were expressed on a multicopy vector (data not shown).

MIC-based assessment of Nisin A, V and T activity against human pathogens

While bioactivity studies with producing strains give a valuable insight into the net consequences of bioengineering lantibiotic genes, they do not differentiate between changes that alter production and those that impact on specific activity. Importantly, previous studies have established that purified Nisin V and Nisin T both exhibit a twofold enhanced specific activity, relative to Nisin A, against *S. aureus* DPC 5245, that Nisin T exhibits twofold enhanced potency against *S. agalactiae* ATCC 13813 and that Nisin V is twofold more potent against *L. monocytogenes* EGDe (Field *et al*., 2008). These data were generated by means of agar diffusion assays using equimolar concentrations of the purified peptides. To ensure that this enhanced activity was not as a consequence of altered diffusion rates in agar, the specific activity of the peptides was assessed against a wide range of targets using classical broth-based minimum inhibitory concentration (MIC) determination assays. Targets included *S. aureus* strains ST 528 (MRSA), ST 530 (MRSA), ST 534 (MRSA), hVISA 32679, hVISA 32652 and DPC5247, VRE strains VRE Ec538, VRE Ec725, VRE Ec533 and VRE Ec748, *C. difficile* ribotype 027, *S. agalactiae* ATCC 13813 and Group B, *S. mutans*, *L. lactis* spp. *cremoris* HP, *Bacillus cereus* as well as *L. monocytogenes* EGDe and 10403S, *L. innocua* FH1848 and the Nisin A resistant mutant *L. monocytogenes* LO28Δ*lisK*. MRSA strains were included given their notoriety in nosocomial and community-acquired infections throughout the world (Zinn *et al*., 2004; Grundmann *et al*., 2006). Investigations established that the MIC of Nisin A with respect to the MRSA strains ST 528, ST 530 and ST 534 was 0.5, 0.5 and 1 mg l^{-1}, respectively (Table 1), which was in close agreement with previous results (Piper *et al*., 2009b). Nisin T was twofold more active than Nisin A against ST 528 and ST 530 (0.26 mg l^{-1} in each case) but was of equal activity against ST 534. In contrast, Nisin V was consistently twofold more potent against all three MRSA indicator strains tested (0.26, 0.26 and 0.52 mg l^{-1} respectively) (Table 1). There was a particular desire to include clinical hVISA and VRE strains as indicators in this study in light of the different means via which these pathogens protect themselves against the lipid II binding activity of vancomycin. hVISA and VISA strains are notable in that they possess a thickened cell wall, which is thought to obstruct access of vancomycin to its molecular target, the

Table 1. Activity of Nisin A, V and T against a range of indicator organisms.

Strain	Nisin A mg l^{-1} (μM)	Nisin V mg l^{-1} (μM)	Nisin T mg l^{-1} (μM)
S. agalactiae ATCC13813	0.13 (0.039)	0.06 (0.019)	0.06 (0.019)
S. agalactiae Group B	0.26 (0.078)	0.13 (0.039)	0.13 (0.039)
S. mutans	8.38 (2.50)	4.19 (1.25)	4.19 (1.25)
C. difficile ribotype 027	8.38 (2.50)	4.19 (1.25)	4.19 (1.25)
L. monocytogenes EGDe	12.57 (3.75)	6.28 (1.875)	12.57 (3.75)
L. monocytogenes 10403S	12.57 (3.75)	6.28 (1.875)	12.57 (3.75)
L. monocytogenes LO28	6.28 (1.875)	ND	ND
L. monocytogenes LO28Δ*lis*K	12.57 (3.75)	6.28 (1.875)	12.57 (3.75)
L. innocua FH1848	12.57 (3.75)	6.28 (1.875)	12.57 (3.75)
S. aureus ST 528 (MRSA)	0.52 (0.156)	0.26 (0.078)	0.26 (0.078)
S. aureus ST 530 (MRSA)	0.52 (0.156)	0.26 (0.078)	0.26 (0.078)
S. aureus ST 534 (MRSA)	1 (0.312)	0.52 (0.156)	1 (0.312)
hVISA 32679[a]	2.51 (0.75)	1.25 (0.375)	1.25 (0.375)
hVISA 32652[a]	2.51 (0.75)	1.25 (0.375)	2.51 (0.75)
S. aureus DPC 5247	0.2 (0.0625)	0.1 (0.0312)	0.1 (0.0312)
E. faecium VRE Ec538[b]	4.19 (1.25)	2.09 (0.625)	4.19 (1.25)
E. faecium VRE Ec725[b]	1.04 (0.312)	0.52 (0.156)	1.04 (0.312)
E. faecium VRE Ec533[b]	2 (0.625)	1 (0.312)	2 (0.625)
E. faecium VRE Ec748[b]	2 (0.625)	1 (0.312)	2 (0.625)
Bacillus cereus	4.19 (1.25)	2.09 (0.625)	4.19 (1.25)
L. lactis spp cremoris HP	0.2 (0.0625)	0.1 (0.0312)	0.05 (0.0156)

a. Heterogenous Vancomycin-intermediate *S. aureus*.
b. Vancomycin-resistant enterococci.
Results given are mean values of three independent determinations.
ND, not determined.

D-Ala-D-Ala motif in the lipid II pentapeptide (Cui *et al*., 2006). hVISA have a vancomycin MIC of ≤ 2 mg l^{-1} but contain a resistant subpopulation able to grow at higher vancomycin concentrations (14 mg l^{-1}) (Howden *et al*., 2006). VRE differ from VISA in that their resistance is not attributed to cell wall thickening, but results from the incorporation of D-Ala-D-Lac in place of D-Ala-D-Ala, resulting in a markedly lower binding affinity for vancomycin (Bugg *et al*., 1991). Studies with purified Nisin A established that the MIC against both hVISA 32679 and hVISA 32652 was 2.51 mg l^{-1} [Table 1; in close agreement with previous data generated with a commercial Nisin A preparation, Nisaplin (Piper *et al*., 2009b)]. These strains were found to be more sensitive to at least one of the bioengineered peptides. While Nisin T was twice as active against hVISA 32679 only (1.25 mg l^{-1}; Table 1), Nisin V exhibited enhanced activity against both strains (1.25 mg l^{-1}; Table 1). Nisin V also outperformed Nisin A and Nisin T when the four VRE isolates (EC 538, EC 533, EC 725 and EC 748) were targeted. The MIC for Nisin V against EC 538, EC 533, EC 748 and EC 725 were 2.09, 1, 1 and 0.52 mg l^{-1}, respectively, which in each case was twofold more active than either Nisin A or Nisin T (Table 1). Another multi-drug resistant clinical pathogen *C. difficile* was also included in our investigations. *Clostridium difficile*-associated diarrhoea (CDAD) is the most frequent hospital-acquired diarrhoea in economically developed countries (Arvand *et al*., 2009). Recently, a hyper-virulent strain designated ribotype (RT) 027 has been implicated in *C. difficile* outbreaks associated with increased morbidity and mortality over the last decade (Arvand *et al*., 2009; Hookman and Barkin, 2009). In MIC experiments conducted with this strain, Nisin A was found to be inhibitory at 8.38 g l^{-1}. However, both Nisin V and Nisin T were twice as active against this target (MIC 4.19 g l^{-1} in both cases). Another, although less virulent, human pathogen is *S. mutans*, which is generally considered to be one of the main etiological agents of dental caries in humans (Hamada and Slade, 1980; Loesche, 1986) and begins to colonize in infants as young as 6 months of age (Wan *et al*., 2003). While this pathogen has been shown to be effectively inhibited by Nisin A (Badaoui Najjar *et al*., 2009), we demonstrate that this activity can be further improved upon in that both Nisin V and Nisin T were twofold more active than Nisin A (4.19, 4.19 and 8.38 mg l^{-1} respectively).

Activity of Nisin derivatives against bovine mastitic pathogens

Because Nisin A has been incorporated into products to prevent or treat bovine mastitis, we chose three representative mastitic isolates for MIC determination studies, including two *S. agalactiae* and one *S. aureus*. This is of particular commercial relevance as mastitis is the most common infectious disease among dairy herds and is estimated to cost US dairy farmers US$1.7 billion annually (Viguier *et al*., 2009), with *S. aureus* being responsible for 15–30% of the infections (Zadoks and Fitzpatrick, 2009). *Streptococcus agalactiae* is also of relevance as it is commonly found in the human gastrointestinal, reproductive and urinary tracts and is the causative agent of Group B streptococcal septicemia, which can be fatal for newborn infants. In line with previous agar-based investigations, Nisin T was found to be twofold more active against *S. agalactiae* ATCC 13813 (0.06 mg l^{-1}) than Nisin A (0.13 mg l^{-1}). Nisin T was also found to be more active against a second representative strain, *S. agalactiae* Group B, with MICs of 0.13 and 0.26 mg l^{-1} for Nisin T and Nisin A respectively. Notably, although agar-based assays had previously indicated that Nisin V and Nisin A possessed similar potency against *S. agalactiae* ATCC13813 (Field *et al*., 2008), broth-based MIC data obtained here established that Nisin V is twofold more active than Nisin A against both *S. agalactiae* targets. This discrepancy could possibly be as a result of the difference between broth based assays compared with solid agar based tests which are known to be affected by several parameters (Wolf and Gibbons, 1996). Having previously established (again through agar-based approaches) that Nisin V and Nisin T exhibit enhanced activity against the bovine mastitis-associated strain *S. aureus* DPC5245 (Field *et al*., 2008), another such isolate, *S. aureus* DPC 5247 (Fitzgerald *et al*., 1997), was employed here. Significantly, several studies indicate that the majority of bovine mastitis cases are caused by only a few specialized clones of *S. aureus* that have a broad distribution (Fitzgerald *et al*., 1997; Zadoks *et al*., 2000; Rabello *et al*., 2005) and thus establishing the sensitivity of another representative of these to the bioengineered Nisin A derivatives is important. MIC analysis revealed that both Nisin V and Nisin T were twofold more active (0.1 mg l^{-1}) than Nisin A (0.2 mg l^{-1}) against *S. aureus* DPC 5247. These results are in agreement with deferred antagonism assays using the *L. lactis* Nisin producing strains (*L. lactis* NZ9800::*nisV* and *L. lactis* NZ9800::*nisT* and *L. lactis* NZ9700) against both mastitis-associated *S. aureus* strains (data not shown).

Activity of Nisin derivatives against food-associated strains

In addition to clinical and veterinary pathogens, a decision was made to investigate the sensitivity of two representative food pathogens. Nisin A is effective against *L. monocytogenes* but it is not very potent (Vignolo *et al*., 2000; Chi-Zhang *et al*., 2004; von Staszewski and Jagus, 2008). Indeed, we also observed that the *Listeria* strains

Table 2. Strains and plasmids utilized in this study.

Strains/plasmids	Relevant characteristics	Reference
Strains		
L. lactis NZ9700	Wild-type Nisin producer	Kuipers *et al*., 1993; 1998
L. lactis NZ9800	*L. lactis* NZ9700Δ*nis*A	Kuipers *et al*., 1993; 1998
L. lactis NZ9800 pVE6007	*L. lactis* NZ9800 harbouring pVE6007	Field *et al*., 2008
L. lactis NZ9800::*nisV*		This study
L. lactis NZ9800::*nisT*		This study
E. coli EC101	*E. coli* host for pORI280	Law *et al*., 1995
Indicator organisms		
Strep. agalactiae	Nisin-sensitive indicator	UCC culture collection
Strep. agalactiae ATCC13813	Nisin-sensitive indicator	American Type Culture Collection
S. aureus DPC5245	Nisin-sensitive indicator	DPC Collection
ST528 (MRSA)	Nisin-sensitive indicator	BSAC
ST530(MRSA)	Nisin-sensitive indicator	BSAC
ST534(MRSA)	Nisin-sensitive indicator	BSAC
hVISA32679	Nisin-sensitive indicator	BSAC
hVISA32652	Nisin-sensitive indicator	BSAC
VRE Ec538	Nisin-sensitive indicator	BSAC
VRE Ec725	Nisin-sensitive indicator	BSAC
VRE Ec533	Nisin-sensitive indicator	BSAC
VRE Ec748	Nisin-sensitive indicator	BSAC
C. difficile ribotype 027	Nisin-sensitive indicator	UCC culture collection
L. monocytogenes 10403S	Nisin-sensitive indicator	UCC culture collection
L. monocytogenes EGDe	Nisin-sensitive indicator	UCC culture collection
L. monocytogenes LO28	Nisin-sensitive indicator	UCC culture collection
L. monocytogenes LO28ΔlisK	Nisin-sensitive indicator	Cotter *et al*., 1999
L. monocytogenes F2365*lux*	Nisin-sensitive indicator	Riedel *et al*., 2007
L. innocua FH1848	Nisin-sensitive indicator	UCC culture collection
B. cereus	Nisin-sensitive indicator	UCC culture collection
L. lactis spp cremoris HP	Nisin-sensitive indicator	UCC culture collection
Plasmids		
pORI280	$RepA^-$, $LacZ^+$	Leenhouts *et al*., 1996
pDF06	pORI280-*nisA*	Field *et al*., 2008
pDF08	pORI280-*nisV*	This study
pDF09	pORI280-*nisT*	This study
pVE6007	Cm^R; temp sensitive	Maguin *et al*., 1992

BSAC, British Society for Antimicrobial Chemotherapy; DPC, Dairy Products Research Centre.

tested here exhibited the greatest natural resistance to Nisin A of all targets tested. This is particularly relevant as this pathogen is the causative agent of listeriosis, one of the most significant foodborne diseases in industrialized countries. Although agar-based analysis had suggested that Nisin V possesses enhanced activity, relative to Nisin A, against two strains of *L. monocytogenes* (EGDe and 10403S) (Field *et al*., 2008), here the activity of both Nisin V and Nisin T were investigated using a broth-based approach and a larger selection of *L. monocytogenes* isolates (Table 2). These investigations revealed that Nisin V is indeed twofold more active (6.28 mg l^{-1}) than Nisin A (12.57 mg l^{-1}) against both *L. monocytogenes* EGDe and 10403S, whereas the activity of Nisin T is equal to that of Nisin A (12.57 mg l^{-1}). This trend was also apparent against a food (fish paste/smoked haddock) isolate, *L. innocua* FH 1848, with the MICs of Nisin V, Nisin T and Nisin A being 6.28, 12.57 and 12.57 mg l^{-1} respectively. Finally, although the development of enhanced resistance by food pathogens to Nisin A has not been reported as a major issue, it remains a concern. Mutants have been generated in the laboratory which exhibit enhanced resistance to Nisin A. One such mutant is *L. monocytogenes* LO28Δ*lisK* (Cotter *et al*., 2002). This mutant lacks the LisK histidine kinase component of the LisRK two-component signal transduction system (Cotter *et al*., 1999; Cotter *et al*., 2002). Here we quantify this resistance and establish that is corresponds to a twofold increase in MIC (LO28, 6.28 mg l^{-1}; LO28Δ*lisK* 12.57 mg l^{-1}). MIC determination studies revealed that while Nisin T again did not exhibit enhanced potency against this *L. monocytogenes* target (12.57 mg l^{-1}), Nisin V is twofold more active (6.28 mg l^{-1}). We can conclude that the mechanism of enhanced resistance mediated by the deletion of *lisK* is overcome by Nisin V. A second food pathogen, *B. cereus*, was also selected for investigation. *Bacillus cereus* is ubiquitous in the environment but is also commonly found in food production settings due to its ability to form biofilms and highly adhesive endospores, allowing it to survive food processing treatments and spread to a variety of foods (Lotte *et al*., 2008). Notably, previous studies have demonstrated that Nisin (in the

form of Nisaplin) was able to inhibit the growth of vegetative *B. cereus* cells in beef gravy at a concentration of 5 mg l^{-1} at 15°C (Beuchat *et al*., 1997). Results from MIC determinations with a *B. cereus* isolate indicate that while the activity of Nisin A and Nisin T are comparable with one another (4.19 mg l^{-1}), Nisin V is twice as potent (2.09 mg l^{-1}). Lastly, due to its high sensitivity to lantibiotics, the cheesemaking strain *L. lactis* spp. *cremoris* HP has been routinely used as an indicator organism in several studies (Cotter *et al*., 2006a,b; Wiedemann *et al*., 2006; Field *et al*., 2007). The MIC of Nisin A against *L. lactis* HP was 0.2 mg l^{-1} (62.5 nM) which is in close agreement to that obtained for Nisin Z (48 nM) (Wiedemann *et al*., 2006). Further analysis with the bioengineered peptide revealed that Nisin V is twofold more active than Nisin A (MIC value of 0.1 mg l^{-1}). In contrast, the Nisin T peptide exhibited a fourfold increase in specific activity compared with Nisin A (0.05 mg l^{-1}).

Growth and kill curve-based comparisons of the activity of Nisin A, T and V

While MIC analysis can illustrate the increased specific activity of the bioengineered peptides compared with Nisin A, they are end-point assays and cannot reveal the more subtle details of the impact of an antimicrobial on bacterial viability that are apparent when growth curve analysis is performed. Furthermore, because such assays are based on the ability of the antimicrobial to retard growth, they do not provide an accurate insight into its ability to kill the pathogen. To address this issue, two of the indicators employed above, *S. agalactiae* ATCC 13813 and *L. monocytogenes* EGDe, were selected for further growth and kill analysis with a view to comparing the results with MIC values. For *S. agalactiae* ATCC 13813, at the concentration of peptide employed (0.04 mg l^{-1}; Fig. 2A), Nisin A caused a slight delay in growth relative to the non-Nisin-containing control. Identical concentrations of the Nisin V and Nisin T resulted in a greater lag time, with the lag time being greatest when Nisin T was employed. Thus while the twofold dilutions of peptide employed for MIC determination did not reveal a difference in the specific activity of Nisin V and Nisin T against *S. agalactiae* ATCC13813, it is apparent from growth curve assays with equimolar concentrations of peptide that Nisin T is, in fact, slightly more potent. Thus while Nisin V is typically more potent than Nisin T, this result coupled with MIC data for *L. lactis* HP, reveals that

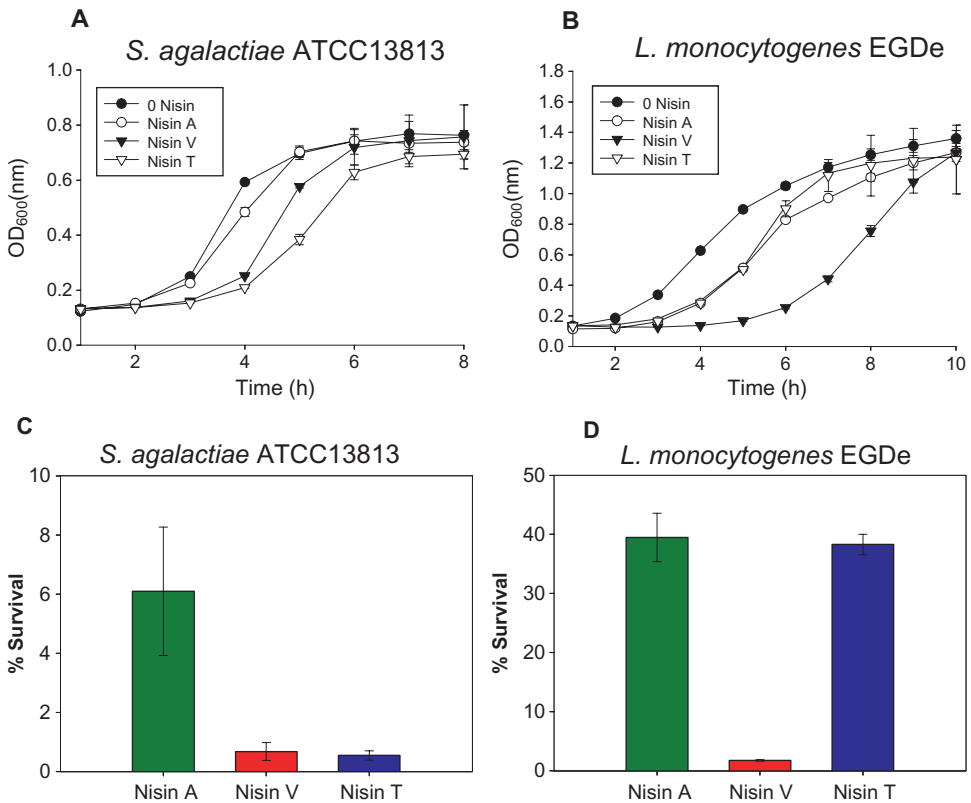

Fig. 2. Growth curve analysis of strains (A) *S. agalactiae* ATCC 13813 and (B) *L. monocytogenes* EGDe in 0.04 mg l^{-1} and 4.19 mg l^{-1}, respectively, of Nisin A, V and T peptides and no peptide (control), and Kill curve analysis of strains (C) *S. agalactiae* ATCC 13813 and (D) *L. monocytogenes* EGDe in 0.1 and 7.5 mg l^{-1} respectively of Nisin A, V and T.

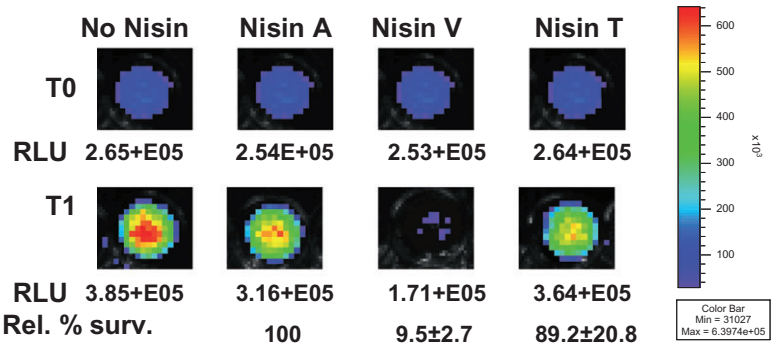

Fig. 3. IVIS imaging of the kill effect of Nisin peptides (7.5 mg l^{-1}) against *L. monocytogenes* F2365*lux* in frankfurter meat over a 1 h exposure period. The top row depicits the image at time 0 (T0). The bottom row depicts the survival of *L. monocytogenes* F2365*lux* after 1 h (T1). The data points represent the corresponding RLU values for images. Rel % Surv figures refer to percentage survival of F2365*lux* as determined by CFU counts after 1 h (T1) where WT Nisin A = 100%.

in some select instances Nisin T is the more active of the two. An investigation of the growth of *L. monocytogenes* EGDe in the presence of sublethal quantities (4.19 mg l^{-1}) of the Nisin peptides (as previously employed by Begley *et al.*, 2006), and a non-Nisin-containing control, highlighted the greater potency of Nisin V as evident from a greatly extended lag. As expected, on the basis of MIC analysis, Nisin A and Nisin T did not differ dramatically (Fig. 2B).

In order to compare the bactericidal activity of Nisin A, Nisin V and Nisin T over a defined period of time, *S. agalactiae* ATCC 13813 and *L. monocytogenes* EGDe were exposed to 0.1 and 7.5 mg l^{-1} concentrations, respectively, of peptide for 1 h. While Nisin A reduced *S. agalactiae* numbers by > 90% after 1 h (6% survival), Nisin V and Nisin T brought about reductions of > 99% (Fig. 2C). Survival rates of 0.675% and 0.543% for Nisin V and Nisin T, respectively, correspond to respective nine- and 11-fold reductions in cell numbers relative to Nisin A. Similar assays with *L. monocytogenes* EGDe reveal that while exposure to Nisin A or Nisin T results in similar cell survival rates (39% and 38%, respectively; Fig. 2D), the same concentration of the Nisin V peptide results in a reduction of > 98% in *L. monocytogenes* EGDe (1.76% survival) i.e. a greater than 20-fold difference in cell counts relative to Nisin A.

Investigation of the anti-Listeria activity of Nisin variants in a food matrix

Having established the potency of Nisin V against *L. monocytogenes* using a variety of laboratory-based assays, we sought to determine whether this enhanced effectiveness could be translated to a food matrix. This was particularly important given that the effectiveness of Nisin A in food can be influenced by a wide range of factors including fat content (Jung *et al.*, 1992; Davies *et al.*, 1999), proteolytic degradation (Murray and Richard, 1997), partitioning into polar or nonpolar food components (Murray and Richard, 1997) and sodium chloride concentrations (Chollet *et al.*, 2008). Thus, to evaluate the efficacy of Nisin V in a situation where Nisin A is traditionally used, the efficacy of Nisin A, Nisin V and Nisin T was compared using frankfurters, a food frequently associated with *L. monocytogenes* contamination, spiked with a strain *L. monocytogenes* F2365 associated with an epidemic outbreak of listeriosis (Linnan *et al.*, 1988; Mascola *et al.*, 1988). Previously, *L. monocytogenes* F2365 was tagged with a luciferase-based reporter system that allows for real-time monitoring in food as well as *in vivo* locations (Riedel *et al.*, 2007). The resultant strain was named F2365*lux*. For food assays, a commercially available frankfurter was homogenized and placed into sterile containers to which *L. monocytogenes* F2365*lux* was added to a concentration of 1×10^7 cfu l^{-1}. Each homogenate of 0.2 ml was transferred to multiwell plates and bioluminescence was quantified using a Xenogen IVIS 100 imager (Time T0). Purified Nisin A, Nisin V or Nisin T peptide was then added to reach a final concentration of 7.5 mg l^{-1}. Following incubation at 37°C for 1 h, bacterial growth was monitored by both bioluminescence imaging (RLU) and plate counts. In the presence of either Nisin A or Nisin T *L. monocytogenes* F2365*lux* numbers increased as indicated by increased bioluminescence from 2.54+E05 relative light units (RLU) and 2.64+E05 RLU to 3.16+E05 RLU and 3.64+E05 RLU, respectively) after 1 h (Fig. 3) whereas in the corresponding Nisin V-treated samples, there was a marked decrease in bioluminescence from 2.53+E05 RLU to 1.71+E05 (Fig. 3). CFU counts after 1 h established that this difference in bioluminescence corresponded to the presence of almost 1 log fewer F2365*lux* cells in the Nisin V-treated frankfurter (9.45 ± 2.7%) relative to the numbers present in the Nisin A- and Nisin T-treated samples [100% and 89.16 ± 20.8% respectively (Fig. 3)]. While these results are in close agreement with the broth-based kill curve experiments described above, they are important in their own right in that they demonstrate that the enhanced potency of Nisin V is maintained even within a complex and high-fat food matrix (total fat content 31.5%). This is reassuring given the problems associated with the inactivity of Nisin in certain foods.

Discussion

The diminished capacity of currently available antibiotics to control unwanted bacteria is a major cause for concern. Due to their many unique properties, the lantibiotic class of bacteriocins would seem to have the potential to breach the gap between effective antibiosis and increasingly drug-resistant clinical and veterinary microbes. Due to its existing broad spectrum activity against a wide range of targets, and its gene-encoded nature, we regard Nisin A as an excellent target for bioengineering-based development to generate more potent microbial inhibitors. The ability to generate these variants in a food-grade manner, as employed here, also provides for the alternative application of these peptides as enhanced food preservatives. Such an application is particularly important in light of the fact that Nisin A has been approved for use, and is widely employed, in over 50 countries worldwide (Chen and Hoover, 2003). Significantly, it has been anticipated that the use of Nisin A is likely to increase in coming years due to the increased consumer demand for minimally processed foods lacking chemical preservatives and the fact that Nisin A is one of only two natural preservatives (the other being the antifungal, natamycin) to have been added to the European Union food additive list. The fact that Nisin V and T differ from Nisin A with respect to only one amino acid suggests that one of these peptides is more likely to be successfully added to this food additive list than any other compound. Such minimal changes also reduce the likelihood of negative consequences such as the peptides becoming cytolytic/hemolytic activity. Significantly Nisin V and T are similar to Nisin A in that no hemolytic activity is apparent even when assessed at very high levels (i.e. 500 mg l^{-1}; data not shown).

Reflecting the multiple ways in which enhanced Nisin derivatives could be applied, the potency of purified Nisin A, V and T was compared against three targets groups; drug-resistant clinical, veterinary and food pathogens. As representative clinical pathogens, four of the most notorious categories of drug resistant pathogens were selected; VRE (four strains), hVISA (two strains), MRSA (three strains) and the fluoroquinolone-resistant *C. difficile* RT 027. The enhanced activity of Nisin V against all 10 of these targets is impressive and not only validates bioengineering strategies for peptide improvement and design, but also serves to highlight the potential of this lantibiotic as an antimicrobial for clinical use. The enhanced efficacy of Nisin V against these targets reveals that the mechanisms *via* which several of these pathogens have developed resistance to vancomycin do not negate the beneficial consequences of the M21V change, thus establishing that enhanced antimicrobial activity is through some as yet unknown mechanism. The second bioengineered peptide, Nisin T, exhibited enhanced activity against only four of these targets and thus the further investigation of this peptide as a novel anti-VRE, hVISA or MRSA compound is not merited at this point. The contrasting, generally enhanced sensitivity of *S. agalactiae* and mastitis infection-associated *S. aureus* to Nisin T provides further evidence that some bioengineered Nisin derivatives exhibit strain-specific enhanced potency. Indeed, given the potency of Nisin V against these mastitis-associated agents it would thus seem that both peptides merit further attention as novel anti-mastitis antimicrobials. Bovine mastitis is the cause of a significant economic cost to dairy enterprises through premature culling, extensive antibiotic treatments, prophylactic antibiotic use, reduction in milk yields and milk wastage. Nisin A is already employed commercially as an anti-mastitis product in the form of Wipe Out ® and recently use of a Nisin A-containing therapeutic, Mast Out ®, was shown to result in highly statistically significant cure-rates in 300 cows with subclinical mastitis (http://www.immucell.com). It has also been suggested that this product may qualify for use in the USA without a requirement to discard milk or withhold meat from human consumption as a consequence of treatment. Thus the existence of bioengineered Nisin derivatives that consistently exhibit enhanced activity against mastitis associated pathogens is noteworthy. However, further animal-trial-based investigations are required to confirm the efficacy of these peptides. From a human medicine perspective, the potential of Nisin in treating infectious mastitis in lactating mothers has already been demonstrated (Fernandez *et al.*, 2008). Significantly, Nisin prepared from the Nis+ strain *L. lactis* ESI 515 effectively reduced staphylococcal numbers in breast milk and led to the complete disappearance of clinical signs of mastitis after 2 weeks of treatment. More importantly, Nisin was successful where therapy using traditional antibiotics had failed to provide any improvement in symptoms (Fernandez *et al.*, 2008).

The enhanced nature of the activity of Nisin V and Nisin T against the hyper-virulent strain *C. difficile* RT 027 is notable. Alternative and novel strategies are urgently needed to treat this increasingly problematic pathogen, especially as reports of resistance to vancomycin (Pelaez *et al.*, 2002) and metronidazole (Brazier *et al.*, 2001) have emerged. Previous examinations of the antimicrobial efficacy of nisin against five strains of *C. difficile* isolated from diseased patients recorded MIC values of between 0.125 mg l^{-1} and 2 mg l^{-1} (Kerr *et al.*, 1997). In this study, the recorded MIC value for Nisin A against *C. difficile* RT 027 was 8.38 mg l^{-1}, which could be due to differences in the methodologies employed or be a reflection of the enhanced stress resistance of the 027 ribotype.

Finally, the growing consumer desire for food that is minimally processed and chemical-free presents new and difficult challenges for the production of fresher and safer food. In this regard Nisin A remains the most commercially important bacteriocin as the only such antimicrobial with GRAS status and WHO, EU and FDA approval. *Listeria monocytogenes* is of particular concern given its widespread distribution in the environment, robustness and ability to grow at refrigeration temperatures. Although the incidence of listeriosis is low, mortality rates associated with outbreaks are high (Schlech, 2000) and consequently several countries worldwide have adopted a zero tolerance in particular foods (Warriner and Namvar, 2009). Therefore, any new technologies or means to enhance the control of *L. monocytogenes* in foods are particularly desirable. While Nisin A has performed this role for decades, Nisin A does not possess very potent anti-*Listeria* activity and thus enhanced derivatives could be employed to increase manufacturer and consumer confidence while still maintaining a 'natural' status. It is thus significant that the Nisin V peptide consistently exhibits superior activity against a range of *L. monocytogenes* isolates. This activity is not diminished in the face of more resistant strains of *Listeria* such as the LO28Δ*lisK* mutant utilized in this study. Moreover, its enhanced efficacy against *L. monocytogenes* F2365*lux* in frankfurter meat indicates it maintains this advantage over wild-type Nisin even within complex food matrices.

From a commercial perspective, it is notable that neither Nisin A nor any other lantibiotic is currently employed commercially as a clinical antimicrobial. However, clinical trials involving duramycin have been reported (Hancock and Sahl, 2006) while mersacidin, among others, are in pre-clinical development (Boakes and Wadman, 2009). In addition, the increasing number of *in vivo* studies using planosporicin, actagardine and microbisporicin demonstrate the potential of lantibiotics for chemotherapeutic exploitation. In addition to the added potency, the use of bioengineered Nisin derivatives as clinical therapeutic agents has additional advantages. One of the major drawbacks associated with novel peptide drugs is the cost of manufacture which can be as high as $100–600 per gram (Hancock and Sahl, 2006). Indeed, although Nisin A was shown to be as effective as penicillin in curing mice infected with *S. pyogenes* and *S. aureus* (Bavin *et al*., 1952) as early as the 1950s, a high cost of production and its rapid clearance *in vivo* were perceived as major obstacles for potential clinical use (Breukink and de Kruijff, 2006). However, due to the use of Nisin A as a food biopreservative, modern large scale fermentation and purification procedures are already in place representing an established base for large scale production of Nisin-like compounds. It is anticipated that these technologies, coupled with the existing awareness of those in the food and veterinary industries of the value of Nisin A as an antimicrobial will be advantageous should novel bioengineered Nisin derivatives be targeted for commercial development.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* strains were grown in M17 broth supplemented with 0.5% glucose (GM17) or GM17 agar at 30°C. *Escherichia coli* was grown in Luria–Bertani broth with vigorous shaking or agar at 37°C. *Staphylococcus aureus* strains were grown in Mueller–Hinton broth (Oxoid) or MH agar at 37°C, streptococci were grown in Tryptic Soy Broth (TSB) or TSB agar at 37°C, *Listeria* strains were grown in Brain Heart Infusion (BHI) or BHI agar at 37°C. VRE were cultured in cation-adjusted Mueller–Hinton broth in accordance with the CLSI microbroth method at 37°C without aeration. Antibiotics were used where indicated at the following concentrations: Chloramphenicol at 10 and 20 μg ml^{-1}, respectively, for *L. lactis* and *E. coli.* Erythromycin was used at 150 μg ml^{-1} and 5 μg ml^{-1} for *E. coli* and *L. lactis* respectively. Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 40 μg ml^{-1}.

Creation of stable Nisin-producing derivatives

Mutagenesis of the *nis*A gene was achieved using a combination of the Quickchange site-directed mutagenesis strategy (Stratagene) and double cross-over mutagenesis with pORI280 (RepA$^-$, LacZ$^+$) as described previously (Cotter *et al*., 2003; 2005b; 2006a; Field *et al*., 2008) using the Quickchange protocol as per manufacturer's guidelines and using *E. coli* EC101 (RepA+) as host. To introduce the desired mutations within the hinge-region of the *nis*A gene, the plasmid pDF06 (a 774 bp product encompassing approx. 300 bp either side of the *nis*A gene cloned into the vector pORI280) was amplified with the QuickChange system (Stratagene) using the primers nisinVFor 5′GAGCTCT GATGGGTTGTAACGTTAAAACAGCAACTTGTCATT3′ and nisinVRev 5′CAATGACAAGTTGCTGTTTTAACGTTACAAC CCATCAGAGCT3′ or nisinTFor 5′CTCTGATGGGTTGTA ACATGACAACAGCAACTTGTCATTGTA3′ and nisinTRev 5′CTACAATGACAAGTTGCTGTTGTCATGTTACAACCCAT CAGA3′ (codon changes underlined). The resulting PCR products were transformed into *E. coli* EC101 (RepA+). To detect altered pORI280-*nis*A transformants, candidates were screened by PCR using a specific 'check' primer i.e. nisinTcheck 5′TGATGGGTTGTAACATGAC and nisinV check 5′GCTCTGATGGGTTGTAACG designed to amplify mutated plasmid template only and a reverse primer oDF106 5′TAGAATTCAACAGACCAGCATTA3′. Plasmids from positive candidates were sequenced (MWG Biotech, Germany) using the primers pORI280FOR 5′CTCGTTCATTATAAC CCTC3′ and pORI280REV 5′CGCTTCCTTTCCCCCCAT3′ to verify the deliberate mutation in each case and to confirm no other changes had been introduced. pDF08 (pORI280-

*nis*M21V) and pDF09 (pORI280-*nis*K22T) were then introduced separately into NZ9800 pVe6007 by electroporation (Holo and Nes, 1995) and transformants were selected by growth on GM17-Ery-Xgal plates at 30°C. Integration of pDF08 and pDF09 by single cross-over recombination and curing of the temperature sensitive plasmid pVe6007 was achieved by growth at 37°C in GM17-Ery broth and plating on GM17-Ery-Xgal agar at the same temperature. Selected colonies were checked for their inability to grow on GM17-Cm agar at 30°C and then subcultured in GM17 at 37°C. Each subculture was spread on GM17-Xgal plates to identify candidates where pORI280 had excised and was lost ($LacZ^-$) due to a second cross-over event. Mutant and wild-type revertants were distinguished by PCR using the specific check primer in each case and oDF106 and also by deferred antagonism assay as candidate mutants exhibited a Bac^+ phenotype and wild-type revertants a Bac^- phenotype. Bac^+ candidates were analysed by Mass Spectrometry to verify production of the mutant Nisin peptide.

Nisin purification

Lactococcus lactis NZ9700 or the mutant Nisin strain of interest was subcultured twice in GM17 broth at 1% at 30°C before use. Two litres of modified TY broth were inoculated with the culture at 0.5% and incubated at 30°C overnight. The culture was centrifuged at 7000 r.p.m. for 15 min. The cell pellet was resuspended in 300 ml of 70% isopropanol 0.1% TFA and stirred at room temperature for approximately 3 h. The cell debris was removed by centrifugation at 7000 r.p.m. for 15 min and the supernatant retained. The isopropanol was evaporated using a rotary evaporator (Buchi) and the sample pH adjusted to 4 before applying to 10 g (60 ml) of Varian C-18 Bond Elut Column (Varian, Harbor City, CA) pre-equilibrated with methanol and water. The columns were washed with 100 ml of 20% ethanol and the inhibitory activity was eluted in 100 ml of 70% IPA 0.1% TFA. Fifteen millilitres of aliquots was concentrated to 2 ml through the removal of propan-2-ol by rotary evaporation. Aliquots (1.5 ml) 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% triflouroacetic acid 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 min at a flow rate of 1.2 ml min^{-1}.

Mass Spectrometry

For Colony Mass Spectrometry (CMS) bacteria were collected with sterile plastic loops and mixed with 50 μl of 70% isopropanol adjusted to pH 2 with HCl. The suspension was vortexed, the cells spun down in a benchtop centrifuge at 14 000 r.p.m. for 2 min, and the supernatant was removed for analysis. Mass Spectrometry in all cases was performed with an Axima CFR plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5 μl aliquot of matrix solution [alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10 mg ml^{-1} in 50% acetonitrile-0.1% (v/v) trifluoroacetic acid] was placed onto the target and left for 1–2 min before being removed. The residual solution was then air-dried and the sample solution (resuspended lyophilized 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 determinations were carried out in triplicate in 96-well microtitre plates. The 96-well microtitre plates were pre-treated with bovine serum albumin (BSA) prior to addition of the peptides. Briefly, to each well of the microtitre plate 200 μl of phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (PBS/BSA) was added and incubated at 37°C for 30 min. The wells were washed with 200 μl of 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. The lyophilized peptides were resuspended in 0.005% acetic acid to a stock concentration of 30 μM. Wild-type Nisin and Nisin mutant peptides were adjusted to a 2.5 μM, 7.5 μM (*Listeria*) or 10 μM (*C. difficile*) starting concentration and twofold serial dilutions of each peptide were made in 96-well plates for a total of 12 dilutions. The target strain was then added and after incubation for 16 h at 30°C or 37°C the MIC was read as the lowest peptide concentration causing inhibition of visible growth.

Clostridium difficile RT 027 was grown anaerobically at 37°C overnight on Fastidious Anaerobic Agar (LabM). A fresh isolated colony was transferred into freshly boiled Reinforced Clostridium Medium (RCM) (Merck) and incubated for ~6 h anaerobically at 37°C. A 1:100 dilution was made into double strength RCM broth, and a 0.1 ml inoculum was added into each well containing the serially diluted nisin peptides (in sterile 50 mM phosphate buffer pH 6.5). The plates were incubated for 20 h in an anaerobic chamber at 37°C and again the MIC read as the lowest peptide concentration causing inhibition of visible growth.

Growth/kill experiments

For peptide Kill assays, fresh overnight cultures were transferred (10^7 cfu ml^{-1} in a volume of 1.0 ml) into TSB-YE or BHI broth containing the relevant concentration of wild-type or mutant Nisin and incubated for 60 min at 37°C. Cell growth was measured by performing viable cell counts by diluting cultures in one-quarter-strength Ringer solution and enumeration on TSB-YE or BHI agar plates. For growth experiments, overnight cultures were transferred (10^7 cfu ml^{-1} in a volume of 1.0 ml) into TSB-YE or BHI supplemented with the relevant concentration of wild-type and mutant Nisins, and subsequently 0.2 ml was transferred to 96-well microtitre plates (Sarstedt). Cell growth was measured spectrophotometrically over 24 h periods by using a Spectra Max 340 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Bioassays for antimicrobial activity

Deferred antagonism assays were performed by replicating strains on GM17 or GM17 Xgal agar plates and allowing them

to grow overnight before overlaying with either GM17/BHI/TS/MH agar (0.75% w/v agar) seeded with the appropriate indicator strain.

Frankfurter meat trial

Thirty-five grams of frankfurter meat (78% pork meat and 12% pork fat) was weighed and placed into a sterile blender and homogenized on full for 30 s with 35 ml of PBS. Meat and meat juices were extracted from the homogenate and placed into sterile 1.5 ml of eppendorf tubes. The samples were then inoculated with approximately 1×10^7 cfu ml^{-1} *L. monocytogenes* F2365*lux*. Samples were then treated with 7.5 μg ml^{-1} of purified Nisin V, Nisin T and Nisin A peptide to achieve final volumes of 1 ml. The kill effect of Nisin against *Listeria* was examined by two techniques: (i) 200 μl of treated homogenate was transferred to 96-well plates and kill of *Listeria* monitored by bioluminescence using the Xenogen IVIS 100 system (Xenogen, ALmeda, CA) with a 2 min exposure time and (ii) 200 μl of treated homogenate was transferred to a sterile eppendorf tube and incubated at 37°C, the number of surviving *Listeria* cells was monitored by serial dilution and plate count technique using *Listeria* Selective Agar (Oxoid). The effect of Nisin against *Listeria* was monitored at time 0 and after 1 h. The test was conducted in triplicate.

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