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In vivo activity of Nisin A and Nisin V against *Listeria monocytogenes* in mice

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Abstract

Background: Lantibiotics are post-translationally modified antimicrobial peptides, of which nisin A is the most extensively studied example. Bioengineering of nisin A has resulted in the generation of derivatives with increased *in vitro* potency against Gram-positive bacteria. Of these, nisin V (containing a Met21Val change) is noteworthy by virtue of exhibiting enhanced antimicrobial efficacy against a wide range of clinical and food-borne pathogens, including *Listeria monocytogenes*. However, this increased potency has not been tested *in vivo*.

Results: Here we address this issue by assessing the ability of nisin A and nisin V to control a bioluminescent strain of *Listeria monocytogenes* EGDe in a murine infection model.
More specifically, Balb/c mice were infected *via* the intraperitoneal route at a dose of 1×10^5 cfu/animal and subsequently treated intraperitoneally with either nisin V, nisin A or a PBS control. Bioimaging of the mice was carried out on day 3 of the trial. Animals were then sacrificed and levels of infection were quantified in the liver and spleen.

Conclusion: This analysis revealed that nisin V was more effective than Nisin A with respect to controlling infection and therefore merits further investigation with a view to potential chemotherapeutic applications.

Keywords: Antimicrobial, Lantibiotic, Bacteriocin, Peptide engineering, Mutagenesis, Nisin

Background

Lantibiotics are ribosomally synthesized peptides produced by Gram-positive bacteria that frequently exhibit potent antimicrobial activities against other bacteria. Nisin A (nisin) is the most intensively investigated lantibiotic, and was first discovered in 1928 [1]. It has a long history of safe use in the food industry and is approved by the US Food and Drug Administration, by WHO and by the EU (as natural food preservative E234) [2-4]. Nisin exhibits antimicrobial activity against many Gram-positive bacteria, including food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. Extensive post-translational modifications are carried out during the biosynthesis of the active 34 amino acid peptide. Specifically, serine and threonine residues in the pro-peptide region are enzymatically dehydrated to dehydroalanine and dehydrobutyrine (Dha and Dhb), respectively. Lanthionine (Lan) and β-methyllanthionine (MeLan) ring structures are generated through the interaction of cysteine with Dha and Dhb, respectively [5-7] (Figure 1). The N-terminal domain, containing one Lan and two meLan rings (A, B, and C) is linked to the C-terminal intertwined rings (D and E) by a flexible hinge region. The antibacterial activity of nisin is exerted *via* a dual action through the activity of the different domains. The N-terminal domain binds to the pyrophosphate moiety of lipid II, inhibiting its transport to the developing cell wall and therefore interfering with cell wall biosynthesis [8]. This binding also facilitates pore formation by the C-terminal domain within the cell membrane, resulting in the loss of solutes from the bacterial cell [9,10].

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 [11-13]. Currently, a number of lantibiotics are under investigation for clinical use. NVB302, a semi-synthetic derivative of actagardine, is in stage I clinical trials with a view to treat infections caused by the hospital-acquired bacteria *Clostridium difficile* [14].

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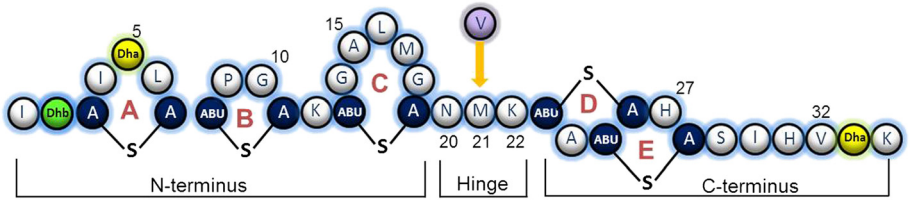


Figure 1 The structure of nisin A showing the location of the N-terminal domain, containing one lanthionine and two (β-methyl) lanthionine rings (A, B, and C) linked to the C-terminal intertwined rings (D and E) by a flexible hinge region. Post-translational modifications are highlighted as follows: dehydroalanine (Dha); dehydrobutyrine (Dhb); lanthionine (A-S-A) and (β-methyl) lanthionine (Abu-S-A). Standard residues are represented in the single letter code. Arrow indicates location of the methionine to valine substitution (M21V) in nisin V.

Similarly, microbisporicin (under the commercial name NAI-107), which targets several multi-drug resistant (MDR) bacteria, is in late pre-clinical trials [15]. In models of experimental infection involving mice and rats, the efficacy of microbisporicin *in vivo* was found to be comparable or superior to reference compounds (vancomycin and linezolid) in acute lethal infections induced with several MDR microbes, including methicillin resistant *Staphylococcus aureus* (MRSA), penicillin-intermediate *Streptococcus pneumonia* and vancomycin resistant enterococci (VRE) [16]. Another lantibiotic, mutacin 1140 (produced by *Streptococcus mutans*) is also undergoing pre-clinical trials [17]. Furthermore, a study involving the two peptide lantibiotic, lacticin 3147, has recently demonstrated its ability to prevent systemic spread of *S. aureus* in a murine infection model [18].

Nisin also displays potent *in vitro* activity against multi-drug resistant pathogens such as MRSA, vancomycin-intermediate and -heterogeneous *S. aureus* (VISA and hVISA, respectively) and VRE, [19-21] while natural variants such as nisin F also show potential in this regard [22]. Notably, several studies have also demonstrated the *in vivo* efficacy of nisin A, [23-25] nisin Z, [26,27] and Nisin F [28,29]. Indeed, nisin F was recently shown to successfully treat respiratory disease caused by *S. aureus* K in immunocompromised Wistar rats [28]. These animals were infected intranasally with 4×10^5 *S. aureus* cells prior to treatment with nisin F, also *via* the nasal route. Furthermore, nisin F was found to control the growth of *S. aureus* for up to 15 minutes in mice when injected into the peritoneal cavity [29]. Animals were dosed with 1×10^8 *S. aureus* cells intraperitoneally and subsequently treated with nisin F, also *via* the intraperitoneal route. In a subsequent study, Nisin F-loaded brushite cement was shown to prevent the growth of *S. aureus* Xen 36 [30]. The brushite cement was subcutaneously implanted into mice and infected with 1×10^3 *S. aureus* cells. Release of nisin F from the bone cement prevented *S. aureus* infection for 7 days.

Despite the potency of nisin and its natural variants, the gene encoded nature of these antimicrobials facilitates bioengineering thereof with a view to enhancing potency [31]. Indeed, bioengineering of the hinge region of nisin A has been particularly successful in generating variants with enhanced potency against Gram-positive pathogens [32,33]. One particular derivative, M21V (also known as nisin V), exhibits an *in vitro* activity against *L. monocytogenes* (the causative agent of listeriosis), and indeed other pathogens, which is superior to that of nisin A [34]. While these laboratory-based studies demonstrate the enhanced potency of nisin V against all Gram-positive bacteria tested thus far, it is not known if this enhancement is also evident *in vivo*. In this study, we address this issue by comparing the efficacy of nisin A and nisin V against a *lux*-tagged strain of *L. monocytogenes* (EGDe::pPL2*lux*pHELP) using a murine infection model and, ultimately, demonstrate the greater efficacy of the bioengineered peptide in controlling infection.

Results/discussion

The ability of nisin A and nisin V to control a *L. monocytogenes* infection in a murine peritonitis model was investigated. Analysis was carried out through bioluminescent imaging of the pathogen in living mice and through the microbiological analysis of organs when mice were sacrificed. Bioluminescence is achieved through the use of a strong constitutive promoter (P_{help} [highly expressed *Listeria* promoter]) driving expression of the *lux* genes of *P. luminescens* integrated into the chromosome of *L. monocytogenes* EGDe [35]. The resulting strain *L. monocytogenes* EGDe::pPL2*lux*pHELP is a strong light-emitter, making it easier to follow *in vivo* using live *in vivo* imaging systems (IVIS). Prior to commencement of the study, the *in vitro* sensitivity of *L. monocytogenes* EGDe::pPL2*lux*pHELP was assessed *via* deferred antagonism assays using nisin A and nisin V producing strains and classical broth-based minimum inhibitory concentration assays (MIC) using purified peptide in each case. Results of deferred antagonism assays with *L. monocytogenes* EGDe::pPL2*lux*pHELP revealed that the nisin V producing strain exhibited increased bioactivity (the combined impact on production and activity) compared to that of *L. lactis* NZ9700 (nisin A producing strain) (Figure 2a). This was in

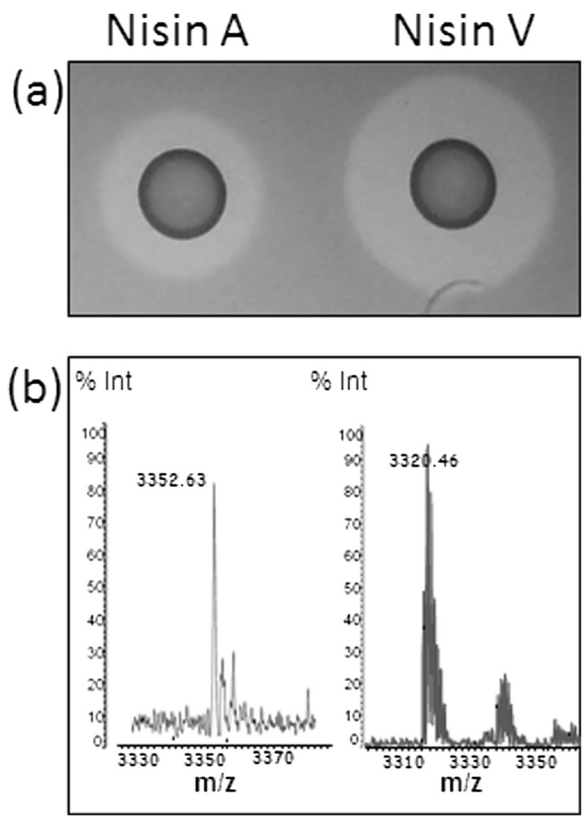


Figure 2 Deferred antagonism assay and mass spectrometry analysis of nisin A and nisin V. (a) Inhibition of growth of *L. monocytogenes* EGDe::pPL2*lux*pHELP by the nisin A producing strain *L. lactis* NZ9700 and the nisin V producing strain *L. lactis* NZ9800*nisA*::M21V. **(b)** Mass spectrometry analysis of the nisin A (3353 amu) and nisin V (3321 amu) peptides produced by the bacterial strains *L. lactis* NZ9700 and *L. lactis* NZ9800*nisA*::M21V, respectively.

close agreement with previous studies highlighting the similar production levels but increased specific activity of nisin V compared to nisin A [32]. Mass spectrometry analysis of purified nisin A and nisin V peptides confirmed that peptides of correct mass were produced (nisin A - 3353 Da; nisin V- 3321 Da) (Figure 2b). The peptides differ by 32 Da, consistent with the methionine21 to valine (M21V) change of the hinge region of the peptide. Following purification, the specific activity of nisin A and nisin V was tested against *L. monocytogenes* EGDe::pPL2*lux*pHELP using minimum inhibitory concentration (MIC) assays. Nisin A was found to be inhibitory at concentrations of 12.57 mg/L (Table 1), which is consistent with the previously established MIC for the non-*lux* tagged parent strain (*L. monocytogenes* EGDe) [34]. Nisin V was found to be two-fold more active against *L. monocytogenes* EGDe::pPL2*lux*pHELP, with an MIC of 6.22 mg/L. Indeed, the superior activity of nisin V was also confirmed against a number of field and clinical strains of *L. monocytogenes*, where nisin V exhibited at least a two-fold improvement against all nisin A-resistant strains (Table 1).

For the *in vivo* study, mice were infected *via* the intraperitoneal route with 1×10^5 cfu of *L. monocytogenes* EGDe::pPL2*lux*pHELP and at 30 minutes post infection were treated intraperitoneally with doses of either nisin A (58.82 mg/kg), nisin V (58.82 mg/kg) or PBS (negative control). On day three of the trial, IVIS imaging was used to quantify the level of infection through the detection of light emitted from the pathogen within the mice (Figure 3). While the initial image suggested that nisin A had reduced the amount of luminescence detected (relative light units or RLU), the difference was not statistically significant compared to the PBS-treated control group (Figure 4a). However, a statistically significant reduction ($P = 0.044$) in RLU measurements was observed in the nisin V treated group when compared to the PBS control group (Figure 4a). These results provide the first evidence of the enhanced *in vivo* efficacy of nisin V relative to nisin A. In addition, microbiological analysis of the liver and spleen was determined after the mice were euthanized. While no statistical difference in listerial numbers was observed in the liver between the nisin A and PBS-containing control groups, average pathogen numbers were significantly lower ($P = 0.018$) by over 1 log in the livers of the nisin V-treated groups (4.70 ± 0.5 log cfu) compared to the control group (6.27 ± 0.25 log cfu) (Figure 4b). Analysis of spleens further highlighted the ability of nisin V with respect to controlling *L. monocytogenes* EGDe::pPL2*lux*pHELP infection. In contrast to the liver-related results, spleen cfu counts revealed that nisin A administration had significantly reduced *Listeria* numbers (5.7 ± 0.17 log cfu) ($P < 0.015$) compared to the control group (6.2 ± 0.2 log cfu) (Figure 4c). However, the number of *Listeria* cells in the spleens of nisin V treated animals was significantly lower again, at 5.1 ± 0.25 log cfu, ($P < 0.015$) than that of the other groups (Figure 4c). While the application of lantibiotics in this way to control *Listeria in vivo* is novel, there have been previous successes with linear non-lantibiotic bacteriocins. Indeed, the class IIA bacteriocins, piscicolin 126 and pediocin PA-1 have been shown to effectively control *L. monocytogenes in vivo* [36,37].

Although, nisin A displays relatively low cytotoxicity towards intestinal epithelial cells *in vitro* [38] and shows no developmental toxicity in rat models [39], the cytotoxicity of nisin V would have to be investigated further before consideration for use in the clinical setting. However, the fact that nisin V lacks haemolytic activity, even at concentrations of 500 mg/L, and differs from nisin A by just one amino acid may mean that a certain amount of read-across will be permitted and a reduced panel of cytoxicity tests could be sufficient to advance

Table 1 *In vitro* activity of nisin A and nisin V against *L. monocytogenes* strains as determined by minimum inhibitory concentration assays[a]

Strain	Equivalent name	Source/Reference	Nisin A mg/L (μM)	Nisin V mg/L (μM)
EGDe::pPL2*lux*pHELP		[35]	12.57 (3.75)	6.22 (1.875)
33028[b]	OB001102	Food	50.28 (15)	24.90 (7.5)
33077[b]	98-18140	Bovine tissue	50.28 (15)	24.90 (7.5)
33225[b]	LMB0455	Unknown	25.14 (7.5)	12.45 (3.75)
F4565[c]	33410, FSLN3-008	Clinical (Los Angeles, California outbreak, 1985)	12.57 (3.75)	6.22 (1.875)
CD1038[d]		Pork sausage	50.28 (15)	12.45 (3.75)

[a]The standard deviation is 0 because of identical triplicate results.
[b]Strain acquired from Todd Ward (Agricultural Research Service, U.S. Department of Agriculture).
[c]Strain acquired from Martin Wiedmann (International Life Sciences Institute).
[d]Strain acquired from Catherine Donnelly (Department of Nutrition and Food Sciences, University of Vermont).

commercial applications. In addition, the success with which bioengineering-based strategies have been employed to enhance its solubility [40], stability [41], diffusion [42] and antimicrobial activity and spectra [32,43,44] would suggest that other derivatives can be generated to further improve upon the functional and pharmokinetic properties of nisin. Alternatively, the use of nisin V in combination with other antimicrobials, such as lysozyme and lactoferrin [28], may also further enhance *in vivo* efficacy.

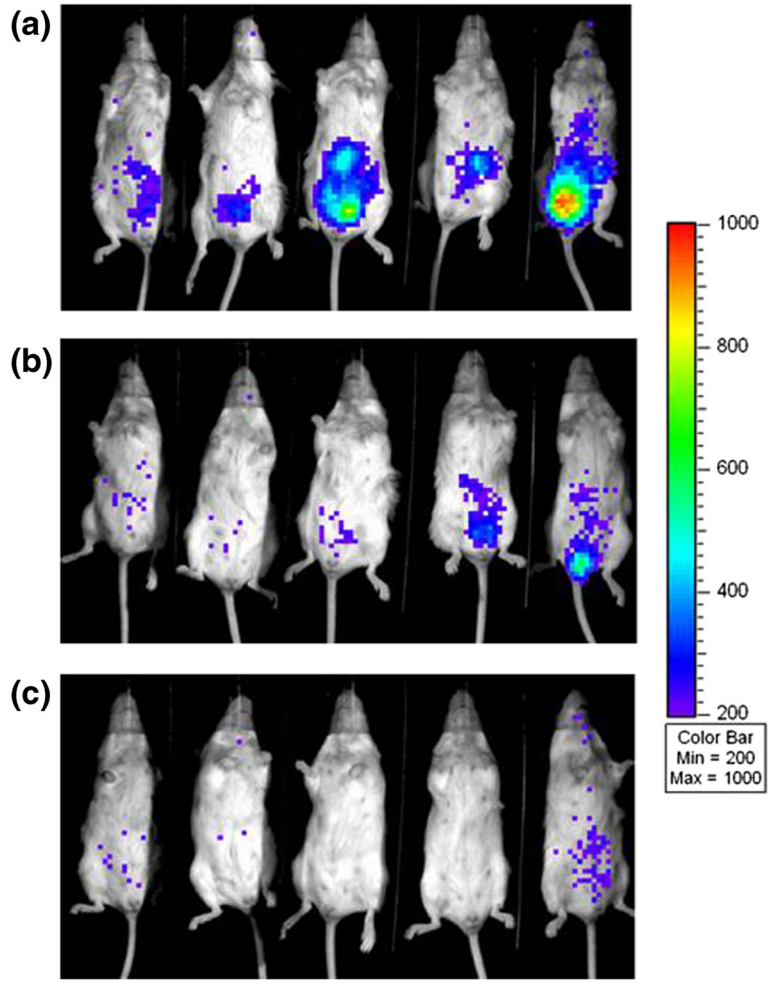


Figure 3 Analysis of effect of nisin A and nisin V on *Listeria* infection in mice 3 days after intraperitoneal infection with 1×10^5 CFU *Listeria monocytogenes* EGDe::pPL2*lux*pHELP. Luminescence observed in animals injected with **(a)** phosphate buffered saline (PBS) **(b)** 58.82 mg/kg nisin A and **(c)** 58.82 mg/kg nisin V 30 minutes after *Listeria* infection.

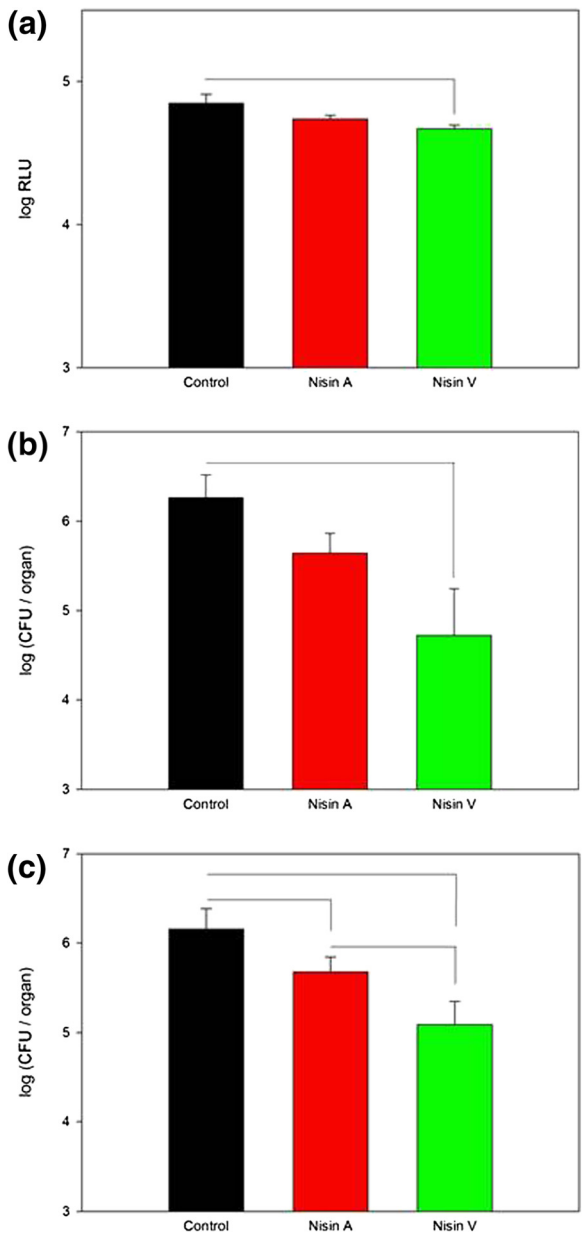


Figure 4 (a) Relative light unit (RLU) counts in mice 3 days after intraperitoneal infection with 1×10^5 CFU *L. monocytogenes EGDe::pPL2luxpHELP*. (b) CFU data from livers and **(c)** spleens of infected mice. Lines connecting groups indicate statistically significant differences between those groups ($P < 0.05$).

Conclusions

This study is the first in which the *in vivo* efficacy of a bioengineered nisin derivative has been assessed. The results revealed that nisin V was more effective than nisin A with respect to controlling infection with *L. monocytogenes* in mice. Significantly, the results validate the use of bioengineering-based strategies for peptide improvement and design and also highlight the potential of nisin V as a chemotherapeutic agent. Enhanced nisins could be especially relevant in situations where traditional antibiotic therapy has failed or where safety issues may predominate. Importantly, the safety of nisin has been well established with, for example, a 90-day oral toxicity study involving rats fed a diet containing nisin A reporting a no-observed-adverse-effect level of approximately 3000 mg/kg/day [45]. Preliminary studies with nisin V revealed a lack of haemolytic activity, even at concentrations of 500 mg/L (D. Field unpublished results).

In conclusion, this study has determined that the enhanced potency of nisin V over nisin A is maintained *in vivo* against the foodborne pathogen *L. monocytogenes* EGDe and suggests that nisin V is a promising candidate as a therapeutic agent.

Methods

Bacterial strains and growth conditions

Lactococcus lactis NZ9700 and *L. lactis* NZ9800*nisA*::M21V strains were cultured in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) and GM17 agar at 30°C. Field isolates of *Listeria monocytogenes* and *Listeria monocytogenes* EGDe::pPL2*lux*pHELP, which harbours the *luxABCDE* operon of *P. luminescens* integrated into the chromosome at a single site [35], was grown in Brain Heart Infusion (BHI) broth (Oxoid) or BHI agar at 37°C.

Nisin purification

Purification of wild type nisin A and the derivative nisin V were carried out as described previously [34]. Briefly, overnight cultures of the wild type nisin A producing strain *L. lactis* NZ9700 [46] and the nisin V producing variant *L. lactis* NZ9800*nisA*::M21V [34] were grown in GM17 broth at 30°C and were subsequently inoculated into two litres of purified TY broth at 1% and incubated overnight at 30°C. The culture was centrifuged at 7,000 r.p.m. for 20 minutes and the supernatant retained. The supernatant was applied to a 60 g Amberlite bead (Sigma) column, which was subsequently washed with 500 ml of 30% ethanol and the inhibitory activity eluted in 500 ml of 70% isopropanol 0.1% trifluoroacetic acid (TFA). The cell pellet was resuspended in 300 ml of 70% isopropanol 0.1% TFA and magnetically stirred for 3 hours at room temperature. The cells were removed by centrifugation at 7,000 r.p.m. for 20 minutes and the supernatant retained. The isopropanol was evaporated off using a rotary evaporator (Buchi) to a volume of 160 ml and the sample pH adjusted to approximately 4.2. The sample was applied to a 10 g (60 ml) Varian C-18 Bond Elut Column previously pre-equilibrated with HPLC water and methanol. The column was washed with 120 ml of 30% ethanol and the inhibitory activity eluted in 60 ml of 70% isopropanol 0.1% TFA. Six millilitres of the lantibiotic preparation was concentrated to 1 ml through the removal of the isopropanol by rotary evaporation and applied to a Phenomenex C12

reverse-phase (RP)-HPLC column, previously equilibrated with 25% isopropanol 0.1% TFA. The column was then developed in a gradient of 30% isopropanol 0.1% TFA to 60% isopropanol 0.1% TFA from 10 to 45 minutes at a flow rate of 2.1 ml/min. Fractions containing nisin A and nisin V peptides were collected and subjected to Mass Spectrometry with a Shimadzu Biotech MALDI-TOF Mass Spectrometer (AXIMA-CFR plus model).

Bioassays for antimicrobial activity

Deferred antagonism assays were carried out as previously described [34]. Briefly, 5 μl of fresh overnight cultures of *L. lactis* NZ9700 and *L. lactis* NZ9800*nisA*::M21V were spotted and allowed to grow on GM17 agar overnight. The colonies were subjected to 30 mins UV radiation prior to overlaying with BHI agar (0.75% w/v agar) seeded with the indicator strain *L. monocytogenes* EGDe::pPL2*lux*pHELP. The plates were then incubated at 37°C overnight and relative zone size compared.

Minimum inhibitory concentration (MIC) assays

The MIC of nisin A and nisin V against *Listeria monocytogenes* EGDe::pPL2*lux*pHELP and several field isolates of *Listeria monocytogenes* was carried out in triplicate as previously described [34]. Briefly, prior to the addition of purified peptides, the 96-well microtitre plates were pretreated with 200 μl of phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) and incubated at 37°C for 30 min. Wells were washed with PBS and left to dry before the addition of 100 μl BHI broth. *L. monocytogenes* strains were grown overnight in BHI broth at 37°C, subcultured into fresh BHI broth and grown to log phase (OD_{600nm} of 0.5). The cultures were diluted to a concentration of 1×10^5 cfu/ml in a 0.2 ml volume. The purified peptides were resuspended in BHI broth to a stock concentration of 60 μM and adjusted to a 15 μM starting concentration. Two-fold dilutions of the peptides were made in the 96-well plates, which were subsequently inoculated with the bacterial strains and incubated at 37°C for 16 hours. The minimum inhibitory concentration (MIC) was read as the minimum peptide concentration inhibiting visible growth of the bacterial strains.

Inoculum preparation

L. monocytogenes EGDe was grown overnight in BHI broth at 37°C from an isolated colony growing on a BHI agar plate containing 7.5 mg/L chloramphenicol. The overnight culture was diluted in order to facilitate its administration in a dose of 1×10^5 cfu/200 μl PBS.

Mouse model

All procedures involving animals were approved by the UCC Animal Experimentation Ethics Committee and carried out by a licensed individual with an ethical approval number of 2011/017. For the *L. monocytogenes* murine model, 15 Balb/c female mice (7 weeks old, 15 g ± 2 g in weight) were divided into three groups (A, B and C) with each group containing 5 mice. At T_0 on day 1, all groups were infected with 1×10^5 viable cells of *L. monocytogenes* EDGe::pPL2*lux*pHELP in a 200 μl dose of PBS *via* the intraperitoneal (I.P.) route. At $T_{0.5hrs}$, mice in group A were administered PBS (control), group B were treated with nisin A (58.82 mg/kg) and group C treated with nisin V (58.82 mg/kg). Both PBS and the nisin peptides were administered in 200 μl doses *via* the I.P. route. On day 3, the mice were anaesthetised using a mixture of aerosolised isoflurane and oxygen. Bioluminescence was monitored using an IVIS® Imaging System 100 series (Xenogen Corporation, Almeda, CA) with a 5 minute exposure time. Immediately afterward, the mice were euthanized and the livers and spleens were extracted. The organs were mechanically disrupted and serial dilutions made which were subsequently plated in 100 μl volumes on BHI agar plates containing chloramphenicol 7.5 mg/L in order to enumerate *L. monocytogenes* present in each organ.

Luminescence quantification

IVIS imaging software was used to carry out quantification of luminescence. Bioluminescence emitted from the infection site was measured as total counts across the region of interest (designated relative light units – "RLU") and was averaged across all groups of mice. The reduction in luminescence was quantified and represents a comparison with the luminescence from mice administered PBS control at the same time point.

Statistical analysis

CFU and RLU data was transformed to $\log_{10}$ prior to analysis. All comparisons were based on the mean ± standard error of the mean (SEM). Parametric data was analysed using one way analysis of variance (ANOVA) with post hoc comparison using the Student-Newman-Keuls method. Non-parametric data was analysed by the Kruskal–Wallis one way ANOVA with post hoc comparison as above. $P < 0.05$ was considered to be significant in all cases.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AC designed experiments, carried out nisin purification, antimicrobial activity bioassays, MIC assays and inoculum preparation and drafted the manuscript. PGC conducted and provided mouse model analysis. DF contributed to the conduct of experiments and reviewing the manuscript. PDC, CH and RPR conceived the study and participated in its design and implementation and reviewed the manuscript. All authors read and approved the final manuscript.

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