Bioengineered Nisin A Derivatives with Enhanced Activity against Both Gram Positive and Gram Negative Pathogens

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Abstract

Nisin is a bacteriocin widely utilized in more than 50 countries as a safe and natural antibacterial food preservative. It is the most extensively studied bacteriocin, having undergone decades of bioengineering with a view to improving function and physicochemical properties. The discovery of novel nisin variants with enhanced activity against clinical and foodborne pathogens has recently been described. We screened a randomized bank of nisin A producers and identified a variant with a serine to glycine change at position 29 (S29G), with enhanced efficacy against S. aureus SA113. Using a site-saturation mutagenesis approach we generated three more derivatives (S29A, S29D and S29E) with enhanced activity against a range of Gram positive drug resistant clinical, veterinary and food pathogens. In addition, a number of the nisin S29 derivatives displayed superior antimicrobial activity to nisin A when assessed against a range of Gram negative food-associated pathogens, including E. coli, Salmonella enterica serovar Typhimurium and Cronobacter sakazakii. This is the first report of derivatives of nisin, or indeed any lantibiotic, with enhanced antimicrobial activity against both Gram positive and Gram negative bacteria.

Introduction

Nisin is the most important commercially exploited member of the heterogeneous family of bacteriocins, antimicrobial peptides produced by bacteria that can kill or inhibit the growth of other bacteria [1]. It is the most highly characterized member of about 60 or so Class 1 bacteriocins, also termed lantibiotics. These are characterized by the presence of post-translational modification unusual amino acids including lanthionine and/or methyllanthionine. These unusual residues are generated by a series of enzyme-mediated modifications that confer a distinct structure and stability. Many lantibiotics, including nisin, lactacin 3147 and mersacidin, are extremely potent and are active against a range of Gram positive targets including antibiotic resistant pathogens [2–6] as well as important food pathogen and spoilage organisms [7,8]. Many lantibiotics are produced by lactic acid bacteria, industrially important food microorganisms that are classified as generally regarded as safe. Several have also been found to function by targeting the essential precursor of the bacterial cell wall, lipid II [9,10], which is also a target for at least four different classes of antibiotic, including the glycopeptide vancomycin. A key advantage of lantibiotics over classical antibiotics is that they are gene-encoded and are thus much more amenable to bioengineering-based strategies with a view to further enhancing their capabilities. Indeed, bioengineering of lantibiotics has been underway for over two decades (for reviews see [11–14]) and has provided a considerable insight into the structure and function of these peptides. It is only in recent years that researchers, armed with a greater understanding of lantibiotic biology and the application of bioengineering strategies on a larger-scale, have achieved notable successes with regard to enhancing the antimicrobial activity of lantibiotics against pathogenic bacteria. Both mersacidin and nukacin have been the subject of comprehensive site-saturation mutagenesis approaches which have resulted in the generation of several novel derivatives with enhanced activity compared to the parent peptide [15,16]. In the case of mersacidin, this included variants with enhanced activity against methicillin resistant Staphylococcus aureus (MRSA), vancomycin resistant enterococci (VRE) and S. pneumoniae. Nisin itself has been subjected to bioengineering for almost twenty years [17–26]. For a comprehensive overview of the data generated by these studies the reader is directed to a number of reviews on the topic ([12–14,27]. Despite the large collection of derivatives which have been generated, relatively few have been found to exhibit enhanced activity against pathogenic microorganisms. An obvious exception relates to derivatives generated by targeting a short 3 amino acid stretch (Asn20-Met21-Lys22) in the centre of the peptide, known as the ‘hinge-region’. Initial success was achieved through the
generation of two mutants, N20K and M21K (Fig. 1), which displayed enhanced activity against Gram negative bacteria including Shigella, Pseudomonas and Salmonella spp. [20]. The generation of nisin derivatives with enhanced activity against Gram positive pathogens was achieved 4 years later using a non-targeted approach [29]. In this instance, the use of a random mutagenesis-based approach to create approximately 8000 nisin derivatives led to the identification of one variant, K22T (Fig. 1), that displayed enhanced activity against Staphylococcus agalactiae, a human and bovine pathogen. Prompted by the identification of yet another ‘hinge’ derivative with enhanced activity, a more extensive ‘hinge-specific’ mutagenesis strategy was undertaken. This led to the identification of further derivatives of note. Four of these were selected for closer inspection [29], with M21V (Fig. 1) being particularly notable by virtue of its enhanced antimicrobial activity against a wide range of targets, including medically significant pathogens such as heterogenous Vancomycin intermediate Staphylococcus aureus (hVISA), VRE, MRSA, Clostridium difficile, S. agalactiae and Listeria monocytogenes [29,30]. This enhanced activity was also apparent in a food setting [30]. Studies with nisin K22T (nisin T) revealed it to be more potent than nisin A against veterinary isolates of S. aureus, S. agalactiae [30] and M. tuberculosis [31] while N20P (nisin P) (Fig. 1) is noteworthy by virtue of the target specific nature of its enhanced activity [29]. More recently, a number of additional nisin ‘hinge’ derivatives have also been identified which exhibit enhanced activity relative to nisin A in complex matrices [32].

Prompted by the success of the aforementioned random mutagenesis approach, we further screened the bank of randomly generated nisin derivatives using additional target species not included in previous studies (S. aureus SA113 and L. monocytogenes LO28). One derivative (S29G) displayed enhanced activity against S. aureus SA113. S29G was subjected to complete saturation mutagenesis to investigate the impact of replacing serine with all 19 other standard amino acids on the bioactivity of nisin. The results reveal the importance of position 29 with respect to the activity of nisin and have for the first time led to the identification of derivatives with enhanced activity against both Gram positive and Gram negative pathogens.

**Materials and Methods**

**Bacterial Strains and Growth Conditions**

The bacterial strains used in this study are listed in Table 1. L. lactis strains were grown in M17 broth supplemented with 0.5% glucose (GM17) or GM17 agar at 30°C. S. aureus strains were grown in Mueller-Hinton (MH) broth (Oxoid) or MH agar at 37°C, streptococci and Bacillus strains 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. C. sakazakii, E. coli and Salmonella strains were grown in Luria-Bertani broth with vigorous shaking or agar at 37°C unless otherwise stated. Antibiotics were used where indicated at the following concentrations: Chloramphenicol at 10 and 20 µg ml⁻¹, respectively for L. lactis and E. coli. Tetracycline was used at 10 µg ml⁻¹ for L. lactis and E. coli.

**Random Mutagenesis**

DNA obtained from L. lactis NZ9700 [33] was used as template for the amplification of a 372 bp fragment encompassing the nisA gene with KOD polymerase (Novagen) using the primers oDF101 and oDF102 (Table 2). PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN Inc.), digested with BglII and XbaI (Roche) and cloned into similarly digested and Shrimp Alkaline Phosphatase (SAP; Roche) treated pPTPL. Following introduction into E. coli MC1000, plasmid was isolated from one clone and was sequenced (MWG Biotech, Germany) using the primer TETK P1 to ensure its integrity. The introduction of this plasmid, pDF03, into competent L. lactis NZ9800 successfully reinstated nisin activity. To provide sufficient quantities of template DNA for error-prone PCR (ep-PCR), nisA was reamplified using pDF03 as template with KOD polymerase

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**Figure 1. Structures of natural nisin and enhanced bioengineered variants.** Six natural variants are known, nisin A, Z, F, Q, U and U2. Black circles indicate amino acid differences between the natural nisin variants. Broken arrows denote enhanced activity of bioengineered nisin A or Z as a result of single amino acid alterations (28,29,52), and/or combination of amino acid substitutions (joined circles) [22]. Residues are represented in the single letter code. Post translational modifications are indicated as follows, Dha: dehydroalanine, Dhb: dehydrobutyrine, Abu: 2-amino butyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: 3-methyl anthionine. Adapted from [14].

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using the primers oDF101 and oDF103, digested with XhoI and EcoR1 and cloned into similarly digested pUC19. Following introduction into E. coli Top 10 (Invitrogen), plasmid was isolated from one clone and was sequenced (MWG Biotech, Germany) using the Maxi-prep plasmid kit (QIAGEN Inc.) to determine if the correct rate of mutation had been achieved. Recombinant plasmid DNA was isolated from the mutant bank was used to transform L. lactis NZ9800. Transformants (approx. 8000) were isolated from Q trays using the Genetix QPIX II-XT colony-picking robot and inoculated into 96 well plates containing GM17 freezing buffer, incubated overnight and subsequently stored at −20°C.

### Saturation Mutagenesis

To generate a template for mutagenesis, the 372 base pair fragment encompassing the nisA gene was amplified with KOD polymerase using the primers oDF102 and oDF103, was digested and subsequently cloned into pCI372. Following introduction into E. coli Top 10 cells, plasmid was isolated from one clone and was sequenced (MWG Biotech, Germany) using the primer pCI372REV to ensure its integrity. Saturation mutagenesis of the serine codon at position 29 of nisA was carried out with pDF05 (pCI372-nisA) as template and using oligonucleotides NisS29degFOR and NisS29degREV (Table 2) containing an NNK codon in place of each native codon. PCR amplification was performed in a 50 μl reaction containing approximately 500 ng of target DNA (pDF05), 1.25 units Mutazyme DNA polymerase, 1 mM dNTPs and 500 ng each of primers oDF102 and oDF103. The reaction was preheated at 96°C for 1 min, and then incubated for 30 secs, 58°C for 1 min and 72°C for 1 min, and then finished by incubating at 72°C for 10 min. Amplified products were purified by gel extraction using the Qiaquick gel extraction kit (QIAGEN Inc), and reamplified with KOD polymerase before being digested with BglII and XhoI (Roche), ligated with similarly digested and SAP treated pPTPL and introduced into E. coli MC1000. To determine if the correct rate of mutation had been achieved recombinant plasmid DNA was isolated from selected clones using the QiAprep Spin miniprep kit (QIAGEN Inc) and sequenced (MWG Biotech). Transformants were pooled and stored in 80% glycerol at −20°C.

### Table 1. Strains used in this study.

| Strains | Relevant characteristics | Reference |
|---------|-------------------------|-----------|
| L. lactis NZ9700 | Wild type Nisin producer | [53,54] |
| L. lactis NZ9800 | L. lactis NZ9700::nisA | [53,54] |
| L. lactis NZ9800pDF05 | L. lactis NZ9800 harboring pCI372 with nisA under its own promoter | [29] |
| L. lactis NZ9800pDF03 | L. lactis NZ9800 harboring pPTPL with nisA under its own promoter | [29] |
| E. coli Top10 | Intermediate cloning host | Invitrogen |
| E. coli MC1000 | E. coli host for pPTPL | [55] |

**Indicator organisms**

- Strep. mitis UCC5001: Nisin sensitive indicator
- S. aureus RF122: Nisin sensitive indicator
- S. aureus Sa113: Nisin sensitive indicator
- ST 528*: Nisin sensitive indicator
- ST 530*: Nisin sensitive indicator
- InVISA 32679*: Nisin sensitive indicator
- L. lactis NZ9800 harboring pPTPL with nisA under its own promoter
- L. lactis NZ9800 harboring pCI372 with nisA under its own promoter
- E. coli MC1000: Nisin sensitive indicator
- L. lactis NZ9800: Nisin sensitive indicator
- L. lactis NZ9800pDF05: Nisin sensitive indicator
- L. lactis NZ9800pDF03: Nisin sensitive indicator

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**Table 1.** Strains used in this study.  
DPC - Dairy Products Research Centre, BSAC – British Society for Antimicrobial Chemotherapy, ATCC – American Type Culture Collection,  
*Methicillin resistant S. aureus.  
#Heterogenous Vancomycin-intermediate S. aureus.  
\[10.1371/journal.pone.0046884.t001\]
Site-directed Mutagenesis to Obtain S29Q

To obtain the last remaining unidentified variant S29Q, site-directed mutagenesis was undertaken using the oligonucleotides nisS29QFor and nisS29QRev (Table 2). Approximately 0.5 ng of the plasmid pDF05 (pCI372-niSA) was used as template for the PCR reaction which was performed in a 50 µl reaction containing 1 unit Phusion High-Fidelity DNA polymerase (Finnzymes, Finland), 1 mM dNTPs and 500 ng each of the appropriate primers pCI372FOR and pCI372REV (Table 2) to verify that mutagenesis had taken place. The purified products were subsequently introduced by electroporation into the strain NZ9800 which has all the genes necessary for nisin production. Subsequently introduced by electroporation into the strain NZ9800 which has all the genes necessary for nisin production. The purified products were then added to 150 µl reaction containing 1 unit Phusion High-Fidelity DNA polymerase (Finnzymes, Finland), 1 mM dNTPs and 500 ng each of the appropriate primers pCI372FOR and pCI372REV (Table 2) to verify that mutagenesis had taken place. The purified products were subsequently introduced by electroporation into the strain NZ9800 which has all the genes necessary for nisin production. Approximately 180 transformants were chosen at random and incubated overnight and stored at 30°C for 20 minutes and the supernatant retained. The 2-propanol was evaporated by rotary evaporator (Buchi) and the sample pH adjusted to 4 before applying to a 10g (60 ml) 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% 2-propanol 0.1% TFA. 15 ml aliquots were concentrated to 2 ml through the removal of 2-propanol by rotary evaporation. 1.5 ml aliquots were applied to a Phenomenex (Phenomenex, Cheshire, UK) C12 reverse phase [RP-HPLC] column (Jupiter 4 u proto 90 Å, 250 x 10.0 mm, 4 µm) previously equilibrated with 25% 2-propanol, 0.1% TFA. The column was subsequently developed in a gradient of 30% 2-propanol containing 0.1% TFA to 60% 2-propanol 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% isopropanol adjusted to pH 2 with HCl. The suspension was vortexed, the cells centrifuged in a benchtop centrifuge at 14,000 r.p.m. 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 0.5 µl aliquot of matrix solution (α-cyano-4-hydroxy cinnamic acid (CHCA), 10 mg ml⁻¹) was applied to a 10g (60 ml) Varian C-18 Bond Elut Column (Varian, Harbor City, CA) pre-equilibrated with 25% 2-propanol, 0.1% TFA. The column was subsequently developed in a gradient of 30% 2-propanol containing 0.1% TFA to 60% 2-propanol containing 0.1% TFA from 10 to 45 minutes at a flow rate of 1.2 ml min⁻¹.

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/BHI/TSB-YE/MH agar (0.75% w/v agar) seeded with the appropriate indicator strain. For higher throughput screening of the S29X 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/BHI/TSB-YE/MH agar (0.75% w/v agar) seeded with the appropriate indicator.

Agarose-based deferred antagonism assays were carried out as follows: GM17/LB (0.03%) underlay was prepared with 10 mM sodium phosphate buffer (SPB) at pH 7.4 to which was added agarose (1% w/v agarose), autoclaved and cooled to 50°C. Bacteria grown to mid-logarithmic phase were harvested by centrifugation and washed with 10 mM SPB at pH 7.4. Bacteria were then added to 15 ml cooled underlay medium to reach a concentration of 2 x 10⁷ colony-forming units (CFU)/ml. The inoculated medium was rapidly transferred into sterile Petri plates, allowed to solidify and dried. Wells (4.6 mm in diameter) were then made in the seeded plates. 10 µl volumes of cell-free supernatant from overnight nisin derivative producing cultures.
or resuspended purified peptides were then added to the wells and the plates incubated at 30°C (L. lactis) or 37°C (Gram positive strains) or RT (Gram negative strains) for 5 hours. Polymyxin B (Sigma Aldrich) was used at a concentration of 20 μg/mL. The plates were then overlaid with 15 ml of autoclaved double strength GM17 (L. lactis) or LB (Gram negatives) agarose (1% w/v agarose) overlay medium precooled to 50°C. The plates were then incubated overnight at the relevant temperature.

Minimum Inhibitory Concentration Assays

Minimum inhibitory concentration determinations for Gram positive organisms were carried out in triplicate in microtitre plates (Sarstech). 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, inoculated into fresh broth and allowed to grow to an OD600 of 0.2 ml. Wild type nisin and nisin mutant peptides were adjusted to a 10 M (S. mitis, B. cereus), 7.5 μM (L. monocytogenes), 5 μM (hVISA), 2.5 μM (MRSA, E. durans), or 500 nM (L. lactis strains) 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.

Minimum inhibitory concentration determinations for Gram negative strains were carried out in triplicate in 96 well microtitre plates. Briefly, bacteria (E. coli, C. sakazakii and Salmoneil) grown to mid-logarithmic phase were harvested by centrifugation, washed with 10 mM SPB at pH 7.4, and diluted to 2×10^5 colony-forming units (CFU)/mL in SPB. Nisin and nisin derivative peptides were resuspended in sterile HPLC water and 50 μL aliquots were added to wells containing 50 μL of 2×10^5 CFU of bacteria. Plates were incubated at RT for two hours with agitation. Double strength Luria–Bertani (LB) broth (100 g/l LB) was added to the wells and the plates were incubated at RT overnight, the MIC was taken as the lowest concentration at which growth was inhibited.

Results

Screening a Bank of Random Nisin Peptides

Although random mutagenesis approaches have rarely been applied to lantibiotics, the recent randomization of the nisin A structural gene led to the identification of a beneficial change with respect to anti-Gram positive activity [29]. Follow-up site-directed and site-saturation mutagenesis within the same region, corresponding to the ‘hinge’ of the peptide, identified three further beneficial mutations with respect to antimicrobial activity [29]. Given that only two indicators (S. aureus SA113 and L. monocytogenes LO28) were detected (Table 3). To complete the collection, site-directed mutagenesis was employed to create the final derivative, S29Q. Screening of this collection of Ser29 derivatives revealed that a number of derivatives, or the supernatants they produced, exhibited superior activity to nisin A producers in agar-based deferred antagonism assays and agarose-based antimicrobial assays against L. lactis HP (Fig. 3), S. aureus RF122 and S. agalactiae ATCC13815 (data not shown). The bioactivity of the S29G variant generated was enhanced as expected, while three additional variants also exhibited enhanced activity against at least one target. These derivatives produced peptides with S29A, S29D and S29E changes (Fig. 3A), as confirmed by mass spectrometry and DNA sequencing.

These assays also provided valuable information regarding the consequences of incorporating other residues at this location. These consequences can be grouped according to the nature of the newly incorporated residue and, in this instance, were compared in terms of their relative impact on bioactivity against L. lactis HP (Table 4). Nisin A is naturally devoid of aromatic residues, and all bioengineered derivatives in which aromatic residues have been incorporated have displayed reduced antimicrobial activity (I1W, M17W, N20F, N20Y, N20W, M21F, M21Y, M21W, K22F, K22W, V32W, E30W, and N20F/M21L/K22Q [28,37,38]. This pattern is again apparent from our studies in that S29F, S29Y and S29W changes were all found to impact negatively on bioactivity (Table 4).

Nisin A is cationic due to the presence of 5 positively charged residues (Lys12, Lys22, Lys34, His27, His31) and the absence of
negatively charged equivalents. To date, the effect of manipulating the charge of nisin has had variable outcomes. This may be a consequence of the location at which the charge residues are incorporated. For instance, the introduction of negatively charged residues into the hinge region has had a detrimental impact (N20D, N20E, M21E, K22D and K22E; [28,29] whereas the introduction of positively charged residues has had a more beneficial impact on anti-Gram-negative activity (N20K and M21K; [28]. Given the importance of positive charge for the initial attraction of many cationic peptides to the cell envelope, it was surprising to find that the introduction of negatively charged residues (S29D, S29E) resulted in the corresponding peptides exhibiting superior bioactivity. S29D and S29E derivatives also displayed higher bioactivity than that of wild type nisin A producer against \( L. \) lactis \( \text{HP} \) (Table 4). In contrast, the replacement of serine 29 with positively charged residues had a negative impact in that the variants with S29R, S29H and S29K changes demonstrated reduced bioactivity.

Replacement of serine with threonine also produced a peptide with a moderate reduction in activity. The mass of this peptide was consistent with the presence of threonine in its unmodified form (3366.75 Da).

In the past, the introduction of hydrophobic residues have had varied impacts on the bioactivity of nisin. In the case of the hinge-region, the introduction of isoleucine, leucine, and methionine (in the latter case at positions 20 or 22) resulted in decreased bioactivity in the majority of cases. In contrast, the introduction of a proline at Asn20 (N20P) or a valine at Met21 (M21V) resulted in peptides with enhanced activity [29,30]. In the present study, the incorporation of isoleucine, leucine, proline and methionine at position 29 resulted in modest reductions in bioactivity against \( L. \) lactis \( \text{HP} \) (Table 4).

The impact on bioactivity as a result of the incorporation of small and nucleophilic residues has generally been favorable, particularly with respect to the hinge region [29]. As noted above, the introduction of a glycine at position 29 (S29G) increases the bioactivity of the corresponding strain against \( S. \) aureus RF122. However, the bioactivity of this strain against \( L. \) lactis \( \text{HP} \) is comparable to that of nisin A (Table 4) while the incorporation of alanine (S29A) has a more beneficial impact (Table 4). Finally, the

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**Figure 2. Bioactivity and mass spectrometry analysis of nisin A and nisin A S29G.** Growth inhibition of (A) \( S. \) aureus SA113 by the nisin A producing strains NZ9800pPTPL-\text{-nisA} and NZ9800pPTPL-\text{-nisA} S29G (B) Colony Mass Spectrometry analysis of nisin A (3352.63 amu) and the nisin A S29G (3322.97 amu) derivative.
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derivatives S29N and S29Q exhibited 83% and 100% of wild type activity, respectively.

As a consequence of the apparent improved bioactivity of the producers of the nisin A S29G, S29A, S29D and S29E derivatives against at least one target, these four derivatives were purified in order to determine if enhanced bioactivity was attributable to enhanced specific activity.

Specific Activities of Nisin A S29G, S29A S29D and S29E against Gram Positive Microorganisms

Although agar-based assays are commonly used for antibiotic MIC determinations in solid media, a number of drawbacks have been identified that relate to altered diffusion rates. This is especially true for molecules of a more hydrophobic or amphiphillic nature, or ones which interact with the diffusion medium, or suffer degradation or loss of substrate during diffusion [39]. To ensure that the enhanced activity of the selected S29 derivatives was not as a consequence of altered diffusion rates, the specific activity of the peptides was assessed against a wide range of organisms using classical broth-based minimum inhibitory concentration (MIC) determination assays. Targets included the antibiotic resistant S. aureus strains ST 528 (MRSA), ST 530 (MRSA), hVISA 32679, as well as S. aureus RF122, Streptococcus mitis, L. lactis HP and MG1363, Bacillus cereus DPC 6088/6089, Enterococcus durans and L. monocytogenes strains 10403S and LO28.

Using equimolar concentrations of purified peptides, the specific activities of S29G, S29A, S29D and S29E were determined (Table 5). For the purpose of comparison, a M21K derivative of nisin A was also generated and purified. A M21K derivative of nisin Z was previously found to possess enhanced activity against some Gram negative targets. These investigations established that the MIC of nisin A against MRSA strains ST 528 and ST 530 was 0.5 and 0.5 mg/L, respectively (Table 5), which was in close agreement with previous results [3]. Both nisin S29G and S29A were two-fold more active than nisin A against both of these targets (0.26 mg/L in each case). One of the other serine 29 derivatives, S29D, displayed a similarly enhanced specific activity against one of these strains, MRSA ST 528. Furthermore, the S29G and S29A derivatives also exhibited improved specific activity against hVISA 32679, with MIC values of 2 mg/L and 2 mg/L, respectively, compared to an MIC of 4 mg/L for nisin A (Table 5). Against the MSSA (methicillin sensitive S. aureus) strain, S. aureus RF122, both S29G and S29A also outperformed nisin A.

| Amino acid     | Molecular Mass | Predicted | Actual  |
|----------------|----------------|-----------|---------|
| Asparagine N   | 3379.66        | 3379.05   |
| Glutamine Q    | 3393.69        | 3393.23   |
| Cysteine C     | 3368.70        | 3368.73   |
| Glycine G      | 3322.60        | 3322.97   |
| Alanine A      | 3356.64        | 3356.67   |
| Serine S       | 3352.06        | 3352.63   |
| Threonine T    | 3366.66        | 3366.75   |
| Valine V       | 3364.69        | 3364.81   |
| Leucine L      | 3378.71        | 3379.33   |
| Isoleucine I   | 3378.71        | 3378.60   |
| Proline P      | 3362.67        | 3362.73   |
| Methionine M   | 3396.74        | 3396.15   |
| Phenylalanine F| 3412.73        | 3412.94   |
| Tyrosine Y     | 3428.73        | 3429.17   |
| Tryptophan W   | 3451.76        | 3451.61   |
| Aspartic acid D| 3380.64        | 3381.32   |
| Glutamic acid E| 3394.67        | 3395.56   |
| Arginine R     | 3421.74        | 3421.93   |
| Histidine H    | 3402.69        | 3404.15   |
| Lysine K       | 3393.72        | 3394.73   |

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nisin A against DPC 6089 (Table 5), while S29A was also twice as potent as (Table 5).

Enterococci are present in high numbers in food production locations due to its ability to form biofilms and highly adhesive endospores, enabling it to survive food processing and certain treatments which can disrupt the outer membrane of Gram negative bacteria can render them susceptible to nisin. Such negative cell wall acts as a barrier for the cell, restricting the access of the peptide to the cytoplasmic membrane [44]. However, in instances its activity equaled nisin A (Table 5). More specifically, the MIC values for both S29G and nisin A against L. monocytogenes LO28 and S. mitis UCC5001 were 6.28 and 8.38 mg/L, respectively. In contrast, nisin S29A exhibited enhanced specific activity against almost all Gram positive targets tested with MICs for L. monocytogenes LO28 and S. mitis UCC5001 being 3.14 and 4.19 mg/L, respectively.

It has previously been reported that the activity of nisin Z M21K is not enhanced against Gram positive targets [28]. Similarly, a L. lactis producer of M21K was found to display bioactivity comparable to that of a nisin A producing control when tested against the Gram positive targets S. aureus ATCC13813 [29]. Here, MIC-based assays with M21K revealed that its specific activity against L. lactis HP, S. aureus RF122, S. mitis UCC5001 and E. durans is equal to that of nisin A but is reduced relative to nisin A against B. cereus DPC 6088 and DPC 6089.

**Specific Activities of Nisin A S29G, S29A, S29D and S29E against Gram Negative Organisms**

Although nisin A has strong antibacterial activity against Gram positive organisms, the outer membrane (OM) of the Gram negative cell wall acts as a barrier for the cell, restricting the access of the peptide to the cytoplasmic membrane [44]. However, certain treatments which can disrupt the outer membrane of Gram negative bacteria can render them susceptible to nisin. Such treatments include chelating agents such as EDTA [45], sub-lethal heat, osmotic shock and freezing [46]. Furthermore, as noted above, a study involving mutagenesis of the hinge region of nisin Z

(MICs of 0.52, 0.52 and 1.04 mg/L, respectively). However, the ‘charge’ mutants S29D and S29E were less active than wild type against this target (4.19, 2.0 and 1.04 mg/L, respectively). In contrast, S29D and S29E, were found to be particularly active when L. lactis HP was used as the indicator organism. While the MIC for nisin A was 0.2 mg/L and that of both S29G and S29A was 0.1 mg/L, the MICs of S29D and S29E were a mere 0.05 mg/L. This pattern appeared to be strain variable in that when another L. lactis MG1363 strain was employed both S29G and S29D were twice as active (0.4 mg/L) as nisin A whereas in this instance nisin S29A was most potent (four fold more active than nisin A (0.2 and 0.8 mg/L, respectively). In contrast, S29D and S29E were found to be particularly active when another 'charge' mutants S29D and S29E were less active than wild type (MICs of 0.52, 0.52 and 1.04 mg/L, respectively). However, the

**Results are expressed as zone diameter in mm. Bold font denotes activity greater than wild type nisin A. Values in bold reached statistical significance compared to a nisin control (Student’s t-test: P<0.05).**

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### Table 4. Agarose assay results of S29 derivatives against Lactococcus lactis HP.

| Nisin derivative (S29X) | Zone Diameter (mm) | % difference (P value) |
|------------------------|--------------------|------------------------|
| **Hydrophilic : Neutral** |                     |                        |
| Serine (wt)            | 15.2 ± 0.2         | 100                    |
| Threonine (T)          | 12.1 ± 0.9         | 79                     |
| Glutamine (Q)          | 15.3 ±0.1          | 100                    |
| Asparagine (N)         | 12.6 ±1.0          | 83                     |
| Tyrosine (Y)           | 10.6 ±0.6          | 70                     |
| **Hydrophilic : Charged** |                   |                        |
| Aspartate (D) −ve      | 20.3 ±0.8          | 133 (0.006)            |
| Glutamate (E) −ve      | 18.6 ±0.3          | 122 (0.0004)           |
| Arginine (R) +ve       | 11.0 ±0.6          | 72                     |
| Histidine (H) +ve      | 10.9 ±0.3          | 72                     |
| Lysine (K) +ve         | 9.9±1.0            | 64                     |
| **Hydrophobic :**      |                     |                        |
| Alanine (A)            | 16.6 ±0.3          | 109 (0.004)            |
| Valine (V)             | 11.6 ±0.4          | 76                     |
| Glycine (G)            | 15.4 ±0.5          | 101                    |
| Cysteine               | 10.5 ±1.0          | 69                     |
| Leucine (L)            | 12.7 ±0.7          | 84                     |
| Isoleucine (I)         | 9.9 ±1.0           | 65                     |
| Tryptophan (W)         | 12.1 ±1.2          | 79                     |
| Phenylalanine (F)      | 11.0 ±1.0          | 72                     |
| Methionine (M)         | 13.6 ±0.8          | 89                     |
| Proline (P)            | 13.7±1.0           | 89                     |

Interestingly, although the specific activity of S29G was enhanced against almost all Gram positive targets tested, in two instances its activity equaled nisin A (Table 5). More specifically, the MIC values for both S29G and nisin A against L. monocytogenes LO28 and S. mitis UCC5001 were 6.28 and 8.38 mg/L, respectively. In contrast, nisin S29A exhibited enhanced specific activity against all Gram positive targets tested with MICs for L. monocytogenes LO28 and S. mitis UCC5001 being 3.14 and 4.19 mg/L, respectively.

Two other foodborne associated strains were selected for MIC analysis, including isolates of Bacillus cereus and Enterococcus durans. B. cereus is ubiquitous in the environment but is often found in food production locations due to its ability to form biofilms and highly adhesive endospores, enabling it to survive food processing treatments [40]. Enterococci are present in high numbers in food of animal origin [41] and vegetables [42] and are recognized as a frequent cause of nosocomial infections [43]. The efficacies of S29G and S29E were two-fold better against B. cereus DPC 6088 and DPC 6089 (Table 5), while S29A was also twice as potent as nisin A against B. cereus DPC 6089 but displayed four-fold improvement against B. cereus DPC 6088. In contrast, S29G was the only derivative to display two-fold improvement against E. durans 5133, while S29A displayed a four-fold increase in potency to nisin A (0.13 mg/L and 0.52 mg/L, respectively (Table 5).
uncovered two mutants N20K and M21K with enhanced antimicrobial activity against the Gram negative targets *Shigella, Pseudomonas* and *Salmonella* [28]. To assess the nisin S29 derivatives against a selection of Gram negative targets of particular foodborne significance, a modified agarose-based assay was utilized to assess the activity of purified nisin A and the nisin A S29G, S29A, S29D and S29E variants against *C. sakazakii*, *E. coli* and *Pseudomonas* strains only and that the nisin S29D was less active than nisin A against *C. sakazakii* and *Salmonella* strains (Table 6). Corresponding studies revealed that the nisin S29E derivative exhibited increased activity against the *E. coli* and *Salmonella* strains only and that the nisin S29D was less active than nisin A against *E. coli* and *Salmonella* but exhibited activity comparable to that of nisin A against the *C. sakazakii* target (Table 6). In contrast, S29G and S29A displayed enhanced activity against all three Gram negative targets tested. Nisin A S29A consistently exhibited greatest potency in this regard (Table 6).

To further confirm the specific activity of the peptides, and to ensure that these results were not a result of improved solubility in solid agar, broth-based MIC determination assays were also carried out using purified peptides against the same Gram negative targets. The results closely matched the patterns obtained using the agarose-based diffusion assays. More specifically, nisin A M21K exhibited a two-fold increase in specific activity compared to nisin A against all the targets tested (Table 7). Nisin A S29E was more active than nisin A against *E. coli* (25.14 mg/L and 50.28 mg/L respectively) and *Salmonella* (50.28 mg/L and >100 mg/L respectively) but not *C. sakazakii*, while nisin A S29D was more active than nisin A against *C. sakazakii* only (6.28 mg/L and 12.57 mg/L, respectively; Table 7). However, both S29G and S29A were two fold more potent against all three targets. These results establish that S29G and S29A variants differ from all nisin derivatives generated to date in that they exhibit enhanced activity against both Gram positive and Gram negative targets. It was also notable that S29A was the only derivative superior to nisin A against all strains utilized in this study.

Finally, while MIC analyses can demonstrate the increased specific activity of a bioengineered peptide, their end point nature does not provide information regarding the relative bactericidal activity of peptides. To address this, the ability of S29G and S29A to kill Gram negative targets was tested and compared with that of nisin A.

### Table 5. Minimum inhibitory concentration results of nisin derivatives against representative Gram positive strains.

| STRAIN                  | NisinA mg/L (µM) | S29G mg/L (µM) | S29A mg/L (µM) | S29D mg/L (µM) | S29E mg/L (µM) | M21K mg/L (µM) |
|-------------------------|------------------|----------------|----------------|----------------|----------------|----------------|
| ST S28                  | 0.52 (0.156)     | 0.26 (0.078)   | 0.26 (0.078)   | 0.26 (0.078)   | 0.52 (0.15)    | nd             |
| ST S30                  | 0.52 (0.156)     | 0.26 (0.078)   | 0.26 (0.078)   | 0.26 (0.078)   | 0.52 (0.156)   | 0.52 (0.15)    |
| NIVSA 32679             | 4.19 (1.25)      | 2 (0.625)      | 2 (0.625)      | 4.19 (1.25)    | 4.19 (1.25)    | nd             |
| L.mono104035            | 12.57 (3.75)     | 6.28 (1.875)   | 6.28 (1.875)   | 12.57 (3.75)   | 12.57 (3.75)   | nd             |
| L.mono LO28             | 6.28 (1.875)     | 6.28 (1.875)   | 3.14 (0.937)   | 12.57 (3.75)   | 6.28 (1.875)   | nd             |
| L. lactis MG1363        | 0.8 (0.250)      | 0.4 (0.125)    | 0.2 (0.062)    | 0.4 (0.125)    | 0.8 (0.250)    | nd             |
| L. lactis HP            | 0.2 (0.062)      | 0.1 (0.031)    | 0.1 (0.031)    | 0.05 (0.015)   | 0.05 (0.015)   | 0.2 (0.062)    |
| S. aureus RF122         | 1.04 (0.312)     | 0.52 (0.156)   | 0.52 (0.156)   | 4.19 (1.25)    | 2 (0.625)      | 1.04 (0.312)   |
| S. mitis UCC5000        | 8.38 (2.5)       | 8.38 (2.5)     | 4.19 (0.125)   | 16.76 (5.0)    | 4.19 (1.25)    | 8.38 (2.5)     |
| B.cereus DPC 6088       | 8.38 (2.5)       | 4.19 (1.25)    | 2 (0.612)      | 16.76 (5.0)    | 4.19 (1.25)    | 16.76 (5.0)    |
| B. cereus DPC 6089      | 8.38 (2.5)       | 4.19 (1.25)    | 4.19 (1.25)    | 8.38 (2.5)     | 4.19 (1.25)    | 16.76 (5.0)    |
| E. durans S133         | 0.52 (0.156)     | 0.26 (0.078)   | 0.13 (0.039)   | 0.52 (0.156)   | 0.52 (0.156)   | 0.52 (0.156)   |

*Methicillin resistant S. aureus.

Minimum inhibitory concentration results of purified nisin wild type peptide and the S29 derivatives S29G, S29A, S29D, S29E and the hinge derivative M21K against a range of Gram positive indicators. Results are expressed as the mean of triplicate assays.

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### Table 6. Results of agarose gel diffusion assays against representative Gram negative strains.

| STRAIN                  | NisinA mm (% wt/Pvalue) | S29G mm (% wt/P value) | S29A mm (% wt/P value) | S29D mm (% wt/P value) | S29E mm (% wt/P value) | M21K mm (% wt/P value) |
|-------------------------|-------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| *C. sakazakii* DPC 6440 | 9.77±0.17               | 10.79±0.16             | 11.65±0.12             | 10.10±0.19             | 7.98±0.29              | 10.51±0.10             |
| *E. coli* 0157-H7       | 9.81±0.04               | 11.10±0.05             | 11.50±0.05             | 8.27±0.08              | 10.54±0.19             | 10.80±0.01             |
| *Salmonella enterica*   | 9.88±0.11               | 10.94±0.24             | 11.29±0.23             | 8.22±0.16              | 10.77±0.07             | 10.75±0.12             |

Results from agarose gel diffusion assays of nisin and S29 variants using purified peptide (60 µM) against the Gram negative strains *C. sakazakii* DPC 6440, *E. coli* 0157-H7 and *Salmonella enterica* serovar Typhimurium UK1. Values are the mean of triplicate agarose gel diffusion assays. All values in bold reached statistical significance compared to the nisin control (Student’s t-test: P<0.05).

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Using purified peptide in each case, 10 mM sodium phosphate buffer washed C. sakazakii, E. coli and Salmonella strains were diluted to a final concentration of $2 \times 10^5$ cfu ml$^{-1}$ and were exposed to nisin A, S29G and S29A at a concentration of 33 mg/L. Following incubation at 37°C for 1 hour, bacterial growth was monitored through plate counts. In all instances the novel derivatives showed greater activity than wildtype nisin A (Fig. 4).

Assays with the polymyxin B nonapeptide (PMBN) were carried out in order to gain an insight into the basis for the enhanced activity of these derivatives against Gram negative targets. Polymyxin B (PMB) is a cyclic lipodecapiptide produced by bacteria of the genus *Pseudomonas* that acts primarily on the Gram negative cell wall [47], leading to rapid permeability changes in the cytoplasmic membrane and ultimately cell death. Polymyxin B nonapeptide (PMBN) lacks the fatty acid tail of PMB and, although it exhibits poor antimicrobial activity, it retains the ability to effect significant permeabilization of the OM, thus rendering Gram-negative bacteria susceptible to various hydrophobic antibiotics [48]. Notably, nisin A and polymyxin B have been shown to be more effective against Gram negative bacteria when used in combination than when either is used alone [49]. We sought to determine if the nisin derivatives S29G and S29A were more effective than nisin A against Gram negative bacteria in combination with PMBN at a concentration of 20 μg/ml. A substantial synergistic effect was clearly evident (Fig. 5). When used in combination with PMBN, a substantial synergistic effect was clearly evident (Fig. 5) but in this instance no significant difference in efficacy was observed between nisin A or either of the derivatives S29A or S29G. Thus the enhanced activity of S29A and S29G over nisin A is only evident in situations where the OM is intact. These results suggest that the enhanced activity of the nisin derivative peptides against Gram negatives is as a result of an increased ability to traverse the OM relative to the nisin A peptide.

**Discussion**

The ribosomally synthesised nature of lantibiotics and the consequent ability to conduct comprehensive bioengineering strategies provides tremendous potential for the development of more effective antimicrobials for food and medical applications. In this study, the screening of a randomly mutated bank of nisin derivatives produced a variant with superior activity against the strain *S. aureus* SA113. The increased efficacy resulted from a single mutation located at serine 29, within the C-terminal of nisin. The importance of Serine 29 for activity has previously been noted when Chan *et al* reported that the removal of five or nine residues

| STRAIN                  | NisinA mg/L (μM) | S29G mg/L (μM) | S29A mg/L (μM) | S29D mg/L (μM) | S29E mg/L (μM) | M21K mg/L (μM) |
|-------------------------|------------------|----------------|----------------|----------------|----------------|----------------|
| *C. sakazakii* DPC 6440 | 12.57(3.75)      | 6.28(1.875)    | 6.28(1.875)    | 6.28(1.875)    | 12.57(3.75)    | 6.28(1.875)    |
| *E. coli* 0157-H7       | 50.28 (15)       | 25.14(7.5)     | >100(>30)      | >100(>30)      | 25.14(7.5)     | 25.14(7.5)     |
| *Salmonella enterica*   |                  |                |                |                |                |                |
| Serovar Typhimurium UK1 | >100 (>30)       | 50.28 (15)     | 50.28 (15)     | >100(>30)      | 50.28 (15)     | 50.28 (15)     |

Minimum inhibitory concentration assays of purified nisin wild type and the derivatives S29G, S29A, S29D, S29E and the hinge variant M21K against the Gram negative strains *C. sakazakii* DPC 6440, *E. coli* 0157-H7 and *Salmonella enterica* serovar Typhimurium UK1. Results are expressed as the mean of triplicate assays.

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Table 7. Minimum inhibitory concentrations of nisin derivatives against representative Gram negative strains.

Figure 4. Kill curve analysis of strains *C. sakazakii* DPC 6440, *S. typhimurium* UK-1 and *E. coli* in 33mg/L respectively of nisin A, S29A and S29G.

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Figure 5. Activity of purified peptides against *C. sakazakii* DPC 6440. Activity of purified peptides of nisin A, S29G and S29A (30 μM) against *C. sakazakii* DPC 6440 as determined by agarose gel diffusion assay using (A) peptide alone and (B) peptide in combination with polymyxin B nonapeptide (PMBN) at a concentration of 20 μg/ml. Results are expressed as total area of inhibitory zone expressed in mm². Values in bold reached statistical significance compared to the nisin control (Student’s t-test: P < 0.05).

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**Nisin S29 Derivatives**

The importance of Serine 29 for activity has previously been noted when Chan *et al* reported that the removal of five or nine residues...
from the C-terminal residues leads to a 16 fold or 110 fold decrease in bactericidal potency compared with that of intact nisin, respectively [35]. Additionally, Sun et al reported that nisin 1–28 also showed a 100 fold reduced inhibitory activity against \textit{L. lactis} MG1363 [36]. These data pointed to an important role for Serine 29 in the activity of nisin and as a consequence, complete saturation mutagenesis was undertaken to determine the impact on nisin activity by substituting serine 29 with all the other available 19 standard amino acids. The strategy proved successful in that three more derivatives, S29A, S29D and S29E displayed enhanced activity against a range of bacterial targets. It is important to note that this improved activity was strain variable, providing further evidence that nisin derivatives can be generated with distinct target specificities. For example, studies with K22T (nisin T) revealed it to be more potent than nisin A against veterinary isolates of \textit{S. aureus} and \textit{S. agalactiae} [30] but not \textit{Listeria monocytogenes}, while N20P (nisin P) is also striking by virtue of the target specific nature of its enhanced activity [29]. Similarly, S29D and S29E displayed improved activity against a distinct number of species, being particularly active against lactococci (\textit{Lactococcus lactis} HP and \textit{Lactococcus lactis} MG1363). In contrast, S29A was more potent than nisin A against all Gram positive and Gram negative bacterial targets.

While nisin was first approved for use in 1969, its use is likely to increase in the coming years due to the increased customer demand for minimally processed foods lacking artificial or chemical preservatives. A major concern in food safety is the transmission of pathogenic enterobacteriaceae (\textit{Salmonella} spp, \textit{E. coli} 0157:H7, \textit{Shigella} spp) due to their major roles in foodborne illness [8]. While nisin is a potent anti-Gram positive inhibitor, its activity against Gram negative bacteria is poor. However, nisin can be used in combination with other synergistic preservation methods (known as hurdle technology), such as organic acids, low pH, high salt concentrations, chelating agents, modified atmosphere packaging, high hydrostatic pressure and thermal treatments, to enhance anti-Gram negative activity [8]. The superior activity of nisin A S29A compared to nisin A against Gram negatives, together with its enhanced activity against all Gram positive targets, suggests that S29A could find applications as a food preservative.

Nisin is also used in the veterinary industry and has potential as a clinical antimicrobial. Bovine mastitis is the cause of significant economic loss to dairy operations. Annual losses are presently estimated to be approximately $2 billion in the US alone [50]. Nisin A is already employed commercially as an anti-mastitis product in the form of Wipe Out®, and an intramammary infusion product Mast Out®, that are being developed as alternatives to traditional antibiotics. Indeed, the Center for Veterinary Medicine of the FDA has recently declared favourably on the application of nisin for the intramammary treatment for subclinical mastitis. More importantly, cattle would not be subject to a zero milk discard and a zero meat withhold as a consequence of treatment.

Thus the existence of bioengineered nisin derivatives that consistently exhibit enhanced activity against mastitis associated pathogens such as the \textit{S. agalactiae} and \textit{S. aureus} RF122 strains utilized in this study is noteworthy. Furthermore, \textit{Escherichia coli} can cause inflammation of the mammary gland in dairy cows around parturition and during early lactation with striking local and sometimes severe systemic clinical symptoms [51]. The bacterium invades the udder through the teat canal and may cause several cases of death per year in the most severe cases. The enhanced nature of S29A and S29G against Gram negative species such as \textit{E. coli} as well as the major mastitis-associated Gram positive species, implies that these derivatives could also reduce the potential for economic loss as a result of their increased potency and broader target range. Indeed, the synergism of nisin A in combination with the polymyxin B nonapeptide reported here would suggest a potential use for this potent combination to control bovine mastitis and, potentially, other veterinary and clinical infections. However, further study is required to establish the mechanistic basis for the enhanced activity of the S29G and S29A derivatives relative to nisin A. In particular, studies will focus on further investigating the importance of the OM with respect to their superior activity against Gram negative bacteria, for which nisin is usually considered ineffective.

In conclusion, it is apparent that altering residue 29 of nisin A can result in the generation of variants with enhanced antimicrobial activity. In some instances this enhancement varies depending on the target microorganism but in other cases, particularly nisin A S29A and, to a lesser extent, S29G, this enhancement is consistent across a wide range of targets. The fact that this enhancement is apparent against Gram positive and Gram negative targets is particularly novel. Further efforts will focus on determining the mechanistic basis for these enhancements and an assessment of how well these peptides perform in food and in vivo.

\section*{Author Contributions}
Conceived and designed the experiments: CH PDC MB DF. Performed the experiments: DF MB KD FH PO. Analyzed the data: DF MB PO. Contributed reagents/materials/analysis tools: CH RPR. Wrote the paper: DF.

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