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Assessing the ability of nisin A and derivatives thereof to inhibit gram-negative bacteria from the genus *Thermus*

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ABSTRACT

Nisin is a bacteriocin that is globally employed as a biopreservative in food systems to control gram-positive, and some gram-negative, bacteria. Here we tested the bioactivity of nisin A-producing *Lactococcus lactis* NZ9700 and producers of bioengineered variants thereof against representatives of the gram-negative genus *Thermus*, which has been associated with the pink discoloration defect in cheese. Starting with a total of 73 nisin variant-producing *Lactococcus lactis*, bioactivity against *Thermus* was assessed via agar diffusion assays, and 22 variants were found to have bioactivity greater than or equal to that of the nisin A-producing control. To determine to what extent this enhanced bioactivity was attributable to an increase in specific activity, minimum inhibitory concentrations were determined using the corresponding purified form of these 22 nisin A derivatives. From these experiments, nisin M17Q and M21F were identified as peptides with enhanced antimicrobial activity against the majority of *Thermus* target strains tested. In addition, several other peptide variants were found to exhibit enhanced specific activity against a subset of strains.
Key words: nisin, *Thermus*, cheese, pink defect

INTRODUCTION

Nisin is a ribosomally synthesized and post-translationally modified bacteriocin with 34 amino acid residues, produced by certain lactic acid bacteria, including particular strains of *Lactococcus lactis*. Nisin is a member of the type-I lantibiotic family of bacteriocins, as it has a flexible elongated structure and possesses the associated unusual (β-methyl) lanthionine amino acids. Nisin A, Z, Q, and F are natural variants of nisin produced by *Lactococcus lactis* ssp. *lactis*, whereas nisin U, U2, P, and H are produced by *Streptococcus* strains (Field et al., 2015; Silva et al., 2018). Nisin has a broad-spectrum inhibition against many gram-positive bacteria, including foodborne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus* spp., *Enterococcus*, and *Clostridium*. The ability of nisin to bind lipid II at its N terminus and form pores at a C-terminal domain linked through a flexible hinge region is important for the translocation of the C-terminal across the bacterial cell membrane, enabling pore formation and the rapid loss of ions and cytoplasmic solutes (Hsu et al., 2002). These events were subsequently found to be responsible for its activities, such as (1) inhibiting bacteria by forming pores in cytoplasmic membrane, (2) inhibiting the outgrowth of bacterial spores by (3) inhibiting cell wall biosynthesis, and (4) inhibiting the activity of structure-dependent autolytic enzymes (Wiedemann et al., 2001; Hsu et al., 2002; Breukink et al., 2003).

Nisin is widely used as a biopreservative in food systems. In 1969, the World Health Organization–Food and Agriculture Organization expert committee on milk and milk products accepted nisin as a food additive for use in processed cheese at a concentration of 12.5 mg/kg (EFSA, 2004; WHO and FAO, 2011), whereas the US Food and Drug Administration (FDA) approves its use to 250 mg/kg (Ibarra-Sánchez et al., 2020). It is also included on the European food additive list, where it was assigned number E234, and has been assigned "generally regarded as safe" status by the FDA since 1988 (Gharsallaoui et al., 2016). Bacteriocins as biopreservatives can be used in 3 different ways: (1) inoculation of bacteriocin-producing strains as starter or adjunct cultures, (2) addition of a purified or semipurified form of bacteriocin in food, or (3) use of a fermentate, produced by a bacteriocin-producing strain, as an ingredient in a food (Chen and Hoover, 2003). The use

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of bacteriocin-producing lactic acid bacteria as starter or adjunct cultures in cheese manufacture, and associated advantages, has been recently reviewed (Silva et al., 2018). A few nisin-related examples of note have involved the use of a combination of nisin-producing strains and a nisin-resistant starter in the manufacture of Emmental and Edam cheeses, which resulted in better cheese quality and inhibition of *Clostridium* spp. without affecting the flavor and eye formation (Hurst, 1981), controlling the growth of *L. monocytogenes* using nisin-producing starters in cottage cheese (Ferreira and Lund, 1996; Dal Bello et al., 2012) and Ricotta cheese (Davies et al., 1997), and providing protection against contaminating bacteria such as *Staph. aureus* in semihard cheese (Mínguez et al., 2000). An example of a commercially available form of nisin as semipurified powder is the food preservative Nisaplin (Dupont, Nutrition and Bioscience, UK; Chen and Hoover, 2003), and nisin in such a form is globally used as a preservative in cheeses (Cleveland et al., 2001; Johnson et al., 2018). In addition to cheese, the use of nisin alone or in combination with other treatments to control the contamination of raw milk, skim milk, and raw skim milk, and in meat products, seafood products, and canned vegetables is common and has been extensively reviewed (Sobrino-López and Martín-Belloso, 2008; Gharsallaoui et al., 2016; Ibarra-Sánchez et al., 2020).

To build on the natural variability among nisin peptides, bioengineering has been employed to further enhance the antimicrobial activity, solubility, protease resistance, and pH stability of nisin peptides. More specifically, nisin derivatives have been developed that possess enhanced antimicrobial activities due to amino acid changes at one or more positions within the N terminus (e.g., substitution of threonine at position 2 to serine in nisin Z; lysine, serine, isoleucine to lysine, phenylalanine, isoleucine at positions 3–5 in nisin A; nisin K12A), the hinge region (e.g., nisin A-NMK to AAA and SAA, N20P, M21V, K22T), or the C-terminus [e.g., nisin A-S29G, S29A, S29D, S29E, S29P, S29P-I30V (nisin PV), nisin F-S29P, nisin Z-S29P, nisin Q-S29P] (Field et al., 2015, 2019; Johnson et al., 2018). Although many studies have demonstrated the enhanced activity of nisin derivatives against pathogenic gram-positive bacteria, only a few examples of nisin derivatives show enhanced activity against gram-negative bacteria such as *Shigella flexneri* 51285, *Salmonella* 50311, *Salmonella enterica* serovar Typhimurium UK1, *Pseudomonas aeruginosa* 1010, *Cronobacter sakazakii* DPC 6400, and *Escherichia coli* O157:H7 (Yuan et al., 2004; Field et al., 2012), and, to some extent, against *Helicobacter pylori* (Kim et al., 2003).

The aim of this study was to assess the antimicrobial activity of nisin A and its derivatives against strains from the gram-negative genus *Thermus*. *Thermus thermophilus* is a carotenoid producer that has been found to be present at higher levels in pink-defective cheese, using high-throughput metagenomic sequencing-based approaches (Quigley et al., 2016). Spiking Swiss-type cheese with different strains of *Thermus* resulted in higher levels of redness in cheese samples (Quigley et al., 2016; Yeluri Jonnala et al., 2019). Thus, identification of biopreservatives that can inhibit the growth of *Thermus* could assist in preventing the pink discoloration defect caused by the presence of *Thermus*.

MATERIALS AND METHODS

Castenholz Medium Preparation

Castenholz tryptone yeast extract (**TYE**) medium was chosen for selective enumeration of *Thermus* bacteria. It was prepared by mixing 500 mL of 2× concentrated Castenholz salts with 100 mL of 1% TYE solute (10.0 g of tryptone, 10.0 g of yeast extract dissolved in 1 L of distilled H_2O) and 400 mL of distilled H_2O. The final pH of Castenholz TYE medium was adjusted to pH 7.6 using hydrochloric acid. For preparation of the corresponding agar, 3% (wt/vol) bacteriological agar was added to the final solution and autoclaved at 121°C for a 15-min cycle.

Castenholz salts (2×) contain 0.2 g of nitrilotriacetic acid, 0.12 g of $CaSO_4 \cdot 2H_2O$, 0.2 g of $MgSO_4 \cdot H_2O$, 0.016 g of NaCl, 0.21 g of KNO_3, 1.4 g of $NaNO_3$, 0.22 g of Na_2HPO_4, 2.0 mL of $FeCl_3$ solution (0.03%), and 2.0 mL of Nitsch's trace elements (0.5 mL of H_2SO_4, 2.2 g of $MnSO_4$, 0.5 g of $ZnSO_4 \cdot 7H_2O$, 0.5 g of H_3BO_3, 0.016 g of $CuSO_4$ $5H_2O$, 0.025 g of $Na_2MoO_4 \cdot 2H_2O$, and 0.046 g of $CoCl_2 \cdot 6H_2O$ dissolved in 1 L of distilled water) were added, and final volume was made up to 1 L with distilled H_2O, followed by stirring until all salts were dissolved, and pH was adjusted to 8.2.

Culturing of Thermus

All *Thermus* bacterial strains used in this study were obtained from the German Collection of Microorganisms and Cell cultures (**DSMZ**, Braunschweig, Germany). These strains, *Thermus thermophilus* HB27 (DSMZ 7039), *Thermus scotoductus* SE-1 (DSMZ 8553), *Thermus aquaticus* YT-1 (DSMZ 625), *Thermus oshimai* SPS-17 (DSMZ 12092), *Thermus rehai* RH99-GF7504 (DSMZ 15653), and *Thermus brockianus* GE-1 (DSMZ 104090), were grown using Castenholz TYE medium at 60°C in a shaking incubator for 48 h. For plating, Castenholz medium with 3% bacteriological agar was used and incubated at 55°C for 3 d.

Culturing of Lc. lactis NZ9700 and Bioengineered Lc. lactis NZ9800 ΔnisA

The nisin A wildtype-producing *Lc. lactis* NZ9700 and *Lc. lactis* NZ9800 Δ*nisA* strains used to produce nisin derivatives were grown in M17 broth (Oxoid, Basingstoke, UK) supplemented with 0.5% glucose or GM17 agar at 30°C for overnight. All *Lc. lactis* strains were obtained from the Dairy Products Center culture collection at Teagasc, Ireland. A brief description of the process by which these strains were originally bioengineered to produce nisin A derivatives follows. A *Lc. lactis* strain from which the nisin A structural gene was deleted, *Lc. lactis* NZ9800, was used as a host. Bioengineered forms of *nisA* were introduced on the *Escherichia coli–Lc. lactis* shuttle vector pCI372 to facilitate trans-expression of the gene and the production of nisin derivatives. Following complete plasmid amplification and introduction into the intermediate *E. coli* Top10 host (Thermo Fisher, Ireland), sequence analysis of a pooled bank of pDF05 derivatives confirmed randomization. Following introduction of these variants into *Lc. lactis* NZ9800, further analyses in the form of colony mass spectrometry and gene sequencing were carried out to determine the extent of amino acid substitution at each position. For more details on the generation of bioengineered nisin derivatives in *Lc. lactis* NZ9800, readers are directed to Field et al. (2008).

Agar Diffusion Assays to Determine Bioactivity Levels

Agar diffusion assays were carried out in triplicate using nisin-producing and indicator (*T. thermophilus* HB27 and *T. scotoductus* SE-1) strains. Both the producer and the indicator strains were cultured as per conditions previously described. One milliliter each of overnight cultures of indicators were added to 50 mL of molten Castenholz agar, briefly swirled, and poured. Wells were made using sterile glass pipettes. To generate cell-free supernatant from nisin-producing *Lc. lactis* strains, 10 mL of overnight cultures of the strains were centrifuged at 950 × *g* for 20 min. The cell-free supernatant was transferred to a sterile tube. Then, 50 μL of cell-free supernatant was added to wells previously described. Plates were incubated at 55°C for 3 d, and the zone of clearance was measured.

Nisin Purification

Lactococcus lactis NZ9700 and NZ9800 variant nisin-producing strains of interest were subcultured twice in GM17 broth or GM17 broth containing chloramphenicol at 10 μg/mL, respectively, at 30°C before use. Two liters of modified tryptone yeast broth (where broth was passed through a column containing XAD-16 Amberlite beads (Bio-Rad, Hercules, CA), which permits better recovery of nisin through removal of hydrophobic constituents) was inoculated with the culture at 0.5% and incubated at 30°C overnight. The culture was centrifuged at 3,820 × *g* for 15 min. The culture supernatant was applied to an Econocolumn containing 60 g of Amberlite XAD 16 beads (2.5 × 2 cm, Bio-Rad). The column was washed with 400 mL of 35% ethanol, and the compound of interest was eluted in 400 mL of 70% 2-propanol 0.1% trifluoroacetic acid. The 2-propanol was evaporated using a rotary evaporator (Buchi AG, Flawil, Switzerland) and the sample applied to a 10-g (60-mL) Strata-E C-18 Bond Elut Column (Phenomenex, Macclesfield, UK) pre-equilibrated with 100% methanol followed by 100% water. The column was washed with 100 mL of 25% ethanol, and the compound of interest was eluted in 100 mL of isopropyl alcohol. Ten-milliliter aliquots were concentrated to 3 mL through removal of 2-propanol by rotary evaporation. Two-milliliter aliquots were applied to a Jupiter Proteo (Phenomenex) C12 reverse-phase HPLC column (4 μm, 90 Å, 250 × 10.0 mm, 4 μm) running at 25 to 45% acetonitrile gradient, where buffer A is 0.1% trifluoroacetic acid and buffer B is 100% acetonitrile 0.1% trifluoroacetic acid. Eluent was monitored at 214 nm, and fractions were collected at 1-min intervals. Nisin-containing fractions were assayed for purity using MALDI-TOF mass spectrometry, and purified fractions were lyophilized in a Genevac HT4 lyophilizer (Genevac Ltd., Ipswich, UK).

MALDI-TOF Mass Spectrometry

In all cases MALDI-TOF analysis was performed with an AximaTOF^2 MALDI-TOF mass spectrometry (Shimadzu Biotech, Manchester, UK). A 0.5-μL aliquot of matrix solution [α-cyano-4-hydroxy cinnamic acid, 10 mg/mL in 50% acetonitrile-0.1% (vol/vol) trifluoroacetic acid] was placed onto the target and left for 1 to 2 min before being removed. The residual solution was then air-dried, and the sample solution 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 analyzed in positive-ion reflectron mode.

MIC Assays

Determinations of MIC for gram-negative *Thermus* strains were carried out in triplicate in round-bottom microtiter plates (Corning Inc., Corning, NY). Ninety-six-well microtiter plates were pretreated with BSA be-

fore addition of the peptides. Briefly, to each well of the microtiter plates 200 μL of d.H_2O (filtered through 0.2-μm filter) containing 1% (wt/wt) BSA was added and incubated at 37°C for 30 min. The wells were washed with 200 μL of 1× PBS twice after removing 1% BSA d.H_2O. Molarities of the purified (as per method previously described) nisin A and nisin A variants were calculated based on the weight obtained and molecular weight. From that, a 4× (2 m*M*) stock concentration was prepared in Castenholz medium before addition into wells of 96-well plates. Then, 100 μL of 1.6 mg/L (1.0 m*M*) nisin peptides were added to the first well of each row, bringing it to 0.5 m*M* concentration and 2-fold serial dilutions were performed until a final concentration of 3.26 μg/L (0.000975 m*M*) was achieved. Target strains were grown overnight as per conditions previously described, and 0.5 mL of overnight culture was subcultured into 10 mL of fresh Castenholz medium and allowed to grow to an optical density (OD_{600}) of ∼0.5 at 55°C. Twenty microliters of this subculture were added to 980 μL of medium, and 150 μL of this dilution added to 14.85 mL of growth medium. Then, 100 μL of this dilution was added to each well, resulting in final inoculums of ∼10^5 cfu/mL in 200 μL. The plates were incubated at 55°C for 3 d, and the MIC was determined as the lowest concentration at which no growth was visible.

Statistical Analysis

Statistical analysis was conducted on 3 replicate well-diffusion assays using SPSS Statistics software, version 24 (IBM Corp., Armonk, NY). Mean differences of clearance zone of well-diffusion assays were determined via ANOVA. The post hoc test Dunnett > control (Keppel and Wickens, 2004) was used for pairwise comparison of clearance zones at a level of significance of $P < 0.05$.

RESULTS AND DISCUSSION

Bioactivity of Nisin A and Its Variants Against Gram-Negative Thermus Bacterial Strains

Bioactivity represents the inhibition of growth of target bacterial strains by nisin A or nisin A variant peptides released into cell-free supernatant. It is to be noted that the bioactivity of cell-free supernatant can be influenced by altered diffusion rates, solubility, production, or specific activity. Thus, the cell-free supernatants of nisin A and nisin variant-producing *Lc. lactis* were used to determine the bioactivity of these lantibiotics against *T. thermophilus* HB27 and *T. scotoductus* SE-1 using agar diffusion assays, with the zone of clearance representing the bioactivity of the producing strain.

Notably, the nisin A producer was active against the *Thermus* strains despite their gram-negative cell wall. We compared the bioactivity of producers of bioengineered nisin A variants with changes in the N-terminal domain, C-terminal domain, and hinge region with the producer of wild-type nisin A. Starting with screening the bioactivity of 71 nisin A derivative-producing strains (Supplemental Table S1, http://hdl.handle.net/11019/2356) tested on both *T. thermophilus* and *T. scotoductus*, we observed that 22 variant producers showed bioactivity that was significantly greater than or equal to that of the nisin A producer (Tables 1 and 2). Among the variants tested were those with altered N-terminal domains. Lysine (K) at position 12, which serves as a small flexible region between rings B and C, is one of the 5 positively charged residues (K12, K22, K34, H27, H3) of nisin A. Variant-containing supernatants in which either hydrophilic [threonine (T), serine (S), tyrosine (Y), glutamine (Q)] or hydrophobic [leucine (L), methionine (M), valine (V)] amino acid residues replaced K12 were studied, and it was established that K12T and K12V exhibited significantly greater bioactivity than that of the wild-type nisin A producer against *T. thermophilus* HB27 (Table 1) and *T. scotoductus* SE-1 (Table 2). The activity of nisin A M17Q, where a hydrophobic residue was replaced by the hydrophilic neutral residue glutamine (Q), was assessed, and no enhancement in bioactivity against *Thermus* was observed.

The hinge region of nisin (i.e., asparagine 20, methionine 21, lysine 22) is a flexible stretch linking the N-terminal and C-terminal domains; its structural role is not clearly understood. Here it was established that producers of M21A, M21V (also known as nisin V), VGA, and PAQ (Table 1) exhibit statistically significant enhanced bioactivity against *T. thermophilus* HB27. In contrast, it is the producers of nisin A M21Q, AAK, and VGA that exhibited significantly better activity than the wild-type producer against *T. scotoductus* SE-1 (Table 2).

The C-terminal domain of nisin with histidine (H) at position 27 is one of 5 positively charged residues in nisin, along with lysine (K) at position 12 in the N-terminal domain. Producers of a peptide in which the imidazole H 27 was changed to the polar-neutral glutamine (Q), where the *nisA* gene was altered and *Lc. lactis* synthesized nisin with Q at position 27 instead of H, displayed statistically significantly better bioactivity against *T. thermophilus* HB27 and higher bioactivity than wild type for *T. scotoductus* SE-1. Furthermore, with respect to the C-terminus, producers of a peptide

Table 1. Bioactivity of nisin A-producing *Lactococcus lactis* and its variants against *Thermus thermophilus* HB27; results are expressed as zone diameter, in mm

Position and nature of residue	*Lactococcus lactis* strain	*Thermus thermophilus* HB27	
		Zone diameter (mm)	% difference (*P*-value)
	Nisin A wild type	6 ± 1.7	100
N-terminal domain			
Hydrophilic: neutral	K 12 T	8.93 ± 1.3	148 (0.001)*
	K 12 S	7.50 ± 2.2	125
	K 12 Y	5.67 ± 0.9	94
	M 17 Q	7.97 ± 1.1	132
Hydrophobic	K 12 L	7.20 ± 1.3	120
	K 12 M	7.10 ± 1.5	118
	K 12 V	8.70 ± 0.8	145 (0.004)*
Hinge region			
Hydrophilic: neutral	N 20 S	7.17 ± 0.9	119
	M 21 Q	7.40 ± 1.3	123
	M 21 Y	6.93 ± 0.8	115
Hydrophobic	N 20 P	5.83 ± 1.8	97
	M 21 F	7.52 ± 0.9	125
	M 21 A	9.50 ± 0.9	158 (0.001)*
	M 21 V	8.27 ± 0.9	137 (0.022)*
2 Hydrophobic, 1 hydrophilic	VGA	10.17 ± 1.4	169 (0.0002)*
	PAQ	8.30 ± 1.4	138 (0.02)*
	PGA	6.83 ± 0.8	113
C-terminal domain			
Hydrophilic: neutral and negatively charged	H 27 Q	8.17 ± 0.7	136 (0.031)*
	S 29 D	10.17 ± 1.5	169 (0.0001)*

*Activity greater than that of wild-type nisin A; values reached significance compared with a nisin control ($P < 0.05$).

in which the polar negatively charged aspartic acid (D) was introduced in place of serine 29 showed significantly enhanced activity against both *T. thermophilus* HB27 and *T. scotoductus* SE-1 (Tables 1 and 2). Although other variants, such as K12L, K12S K12M, N20S, M21F, M21Q, M21Y, and PGA, have better an-

Table 2. Bioactivity of nisin A-producing *Lactococcus lactis* and its variants against *Thermus scotoductus* Se-1; results are expressed as zone diameter, in mm

Position and nature of residue	*Lactococcus lactis* strain	*Thermus scotoductus* SE-1	
		Zone diameter (mm)	% difference (*P*-value)
	Nisin A wild type	6.5 ± 0.5	100
N-terminal domain			
Hydrophilic: neutral	K 12 T	9.1 ± 1	140 (0.001)*
	K 12 Y	4.80 ± 0.2	74
	K 12 S	8 ± 0.01	123
Hydrophobic	K 12 V	8.10 ± 0.9	125 (0.04)*
	K 12 M	7.65 ± 1	118
Hinge region			
Hydrophilic: neutral	N 20 S	7.25 ± 0.9	112
	M 21 Q	8.05 ± 1	124 (0.05)*
	M 21 Y	7.50 ± 0.5	115
Hydrophobic	N 20 P	7.50 ± 0.6	115
	M 21 F	6.90 ± 0.8	106
	M 21 V	7.75 ± 0.9	119
	M 21 M	7.65 ± 0.7	118
2 Hydrophobic, 1 hydrophilic	AAK	8.60 ± 0.9	132 (0.004)*
	VGA	8.25 ± 0.3	127 (0.022)*
	VGV	7.45 ± 1	115
C-terminal domain			
Hydrophilic: neutral and negatively charged	H 27 Q	7.50 ± 0.5	115
	S 29 D	8.15 ± 0.6	125 (0.033)*

*Activity greater than that of wild-type nisin A; values reached significance compared with a nisin control ($P < 0.05$).

tibacterial activity than the wild-type producer against *T. thermophilus* HB27, and K12S, K12M, N20P, N20S, M21F, M21Y, M21V, and VGV have higher antimicrobial activity than the wild-type producer against *T. scotoductus* SE-1, these enhancements are not statistically significant.

Specific Activity of Nisin A and Its Variants Against Gram-Negative Thermus

To determine to what extent, the previously described bioactivity is due to increased specific activity, nisin A (control) and its derivatives were purified and their molecular weights confirmed via MALDI-TOF mass spectrometry (Table 3). Nisin A variants that showed significant bioactivity and bioactivity close to that of wild-type nisin A were used to determine MIC against 6 *Thermus* strains: *T. thermophilus* HB27, *T. scotoductus* SE-1, *T. rehai*, *T. oshimai*, *T. aquaticus*, and *T. brockianus*.

The MIC obtained are the means of triplicate experiments tested against the target strains (Table 4; Figure 1). The N-terminal-domain nisin A variants tested were K12T, K12V, K12S, K12Y, K12M, K12L, M17Q. The MIC for nisin A peptide against *T. thermophilus* HB27, *T. oshimai*, and *T. rehai* was 26.1 μg/L, 52.3 μg/L for *T. brockianus* and *T. aquaticus*, and 209.6 μg/L for *T. scotoductus* SE-1. The K12V peptide displayed 2-fold better activity than wild-type nisin A activity (refer to Table 4 for wild-type nisin A MIC) against *T. thermophilus* HB27, *T. scotoductus* SE-1, and *T. rehai* (MIC of 12.9 μg/L, 103.9 μg/L, and 122.9 μg/L, respectively). The M17Q peptide showed better activity against all strains tested, exhibiting 2-fold better activity against *T. brockianus* and *T. aquaticus* (6.5 μg/L and 26.2 μg/L, respectively), 4-fold against *T. scotoductus* SE-1 (52.28 μg/L) and *T. rehai* (6.53 μg/L), and 8-fold better activity against *T. thermophilus* HB27 (3.2 μg/L) and *T. oshimai* (3.2 μg/L). The variant K12S showed 2-fold better activity against *T. scotoductus* SE-1 (103.5 μg/L) only. Variant K12Y displayed 2-fold and 4-fold better activity against *T. aquaticus* (26.4 μg/L) and *T. rehai* (6.6 μg/L) respectively, whereas K12L was 2-fold (26 μg/L) more active than nisin A against *T. aquaticus*.

Table 3. Analysis of nisin A and its variants used in this study by MALDI-TOF mass spectrometry for MIC assays

Strain	Molecular mass	
	Predicted	Obtained
Nisin A wild type	3,354.07	3,353.55
AAK	3,250.92	3,251.59
K12T	3,326.99	3,326.63
K12V	3,325.03	3,324.33
M21V	3,322.02	3,321.52
K12S	3,312.97	3,312.58
N20P	3,337.08	3,336.70
S29D	3,382.09	3,381.61
M21A	3,293.96	3,291.17
N20S	3,327.05	3,325.52
M21F	3,370.05	3,368.29
K12Y	3,389.07	3,388.32
PAQ	3,276.93	3,276.93
M21Y	3,385.66	3,384.70
M21Q	3,350.61	3,350.64
VGA	3,207.863	3,205.30
K12L	3,339.05	3,337.98
H27Q	3,342.67	3,344.14
PGA	3,205	3,203.79
K12M	3,357.07	3,357.32
M17Q	3,351.07	3,349.18

Hinge-region nisin A variants N20P, N20S, M21V, M21A, M21F, M21Y, M21F, AAK, PAQ, VGA, and PGA were compared with nisin A against target strains. We found that N20S exhibited enhanced activity (2- to 8-fold) against 5 of the targeted strains, the exception being *T. brockianus*. The introduction of polar-neutral residues [tyrosine (Y), glutamine (Q)] at position M21 resulted in enhanced activity against *T. oshimai*, *T. rehai*, and *T. aquaticus* (MIC for M21Y were 13.2 μg/L, 13.2 μg/L, and 6.6 μg/L, and those for M21Q were 6.5 μg/L, 13 μg/L, and 26.1 μg/L, respectively), whereas M21Y was 2-fold more active against *T. thermophilus* HB27 (13.2 μg/L). Variant M21F showed better antimicrobial activity against all of the target strains, with a 2- to 8-fold difference compared with wild type. Aromatic residues are absent from the nisin A peptide, and with incorporation of phenylalanine (F), the enhanced activity of M21F against the 6 targets is notable.

The C-terminal domain nisin A variants tested were H27Q and S29D. Of these, H27Q showed an 8-fold enhanced activity against *T. thermophilus* HB27, *T. oshimai*, and *T. rehai* (3.2 μg/L) and 14-fold enhanced activity against *T. aquaticus* (3.26 μg/L).

DISCUSSION

Nisin is a ribosomally synthesized bacteriocin that is approved for use as a food biopreservative by many agencies. It exhibits a broad spectrum of bactericidal activity against many gram-positive bacteria, including foodborne pathogens, and its mode of action involves the creation of pores in the cell membrane or the inhibition of peptidoglycan synthesis by preventing transpeptidation, or both. In this regard, the importance of the lipid II molecule has been well studied (Brötz et al., 1998; Wiedemann et al., 2001; Breukink et al., 2003), where the N-terminal domain of nisin is important for binding with the pyrophosphate moiety of lipid II to mediate pore formation by the C-terminal domain in the cell

Table 4. Minimum inhibitory concentration results of purified nisin A wild type and its variants against gram-negative *Thermus* bacterial strains; results are expressed as means of triplicate assays

	MIC $\mu g/L_{(mM)}$					
Nisin peptide	*Thermus thermophilus* HB27	*Thermus scotoductus* SE-1	*Thermus oshimai*	*Thermus rehai*	*Thermus brockianus*	*Thermus aquaticus*
Nisin A wild type	$26.16_{(0.0078)}$	$209.63_{(0.0625)}$	$26.16_{(0.0078)}$	$26.16_{(0.0078)}$	$52.32_{(0.0156)}$	$52.32_{(0.0156)}$
K12S	$103.53_{(0.0312)}$	$103.53_{(0.0312)}$[1]	$51.68_{(0.0156)}$	$51.68_{(0.0156)}$	$103.53_{(0.0312)}$	$103.53_{(0.0312)}$
K12T	$51.90_{(0.0156)}$	$207.94_{(0.0625)}$	$103.97_{(0.0312)}$	$51.90_{(0.0156)}$	$207.94_{(0.0625)}$	$103.97_{(0.0312)}$
K12V	$12.97_{(0.0039)}$[1]	$103.91_{(0.0312)}$[1]	$51.87_{(0.0156)}$	$12.97_{(0.0039)}$[1]	$51.87_{(0.0156)}$	$51.87_{(0.0156)}$
K12L	$52.09_{(0.0156)}$	$208.69_{(0.0625)}$	$26.04_{(0.0078)}$	$26.04_{(0.0078)}$	$104.35_{(0.0312)}$	$26.04_{(0.0078)}$[1]
K12Y	$52.87_{(0.0156)}$	$211.82_{(0.625)}$	$52.87_{(0.0156)}$	$6.61_{(0.00195)}$[1]	$105.91_{(0.0312)}$	$26.43_{(0.0078)}$[1]
K12M	$52.37_{(0.0156)}$	$419.63_{(0.125)}$	$52.37_{(0.0156)}$	$26.19_{(0.0078)}$	$209.82_{(0.0625)}$	$52.37_{(0.0156)}$
M17Q	$3.27_{(0.000975)}$[1]	$52.28_{(0.0156)}$[1]	$3.27_{(0.000975)}$[1]	$6.53_{(0.00195)}$[1]	$26.14_{(0.0078)}$[1]	$26.14_{(0.0078)}$[1]
N20S	$12.98_{(0.0039)}$[1]	$103.97_{(0.3125)}$[1]	$3.24_{(0.000975)}$[1]	$6.49_{(0.00195)}$[1]	$51.90_{(0.0156)}$	$25.95_{(0.0078)}$[1]
N20P	$52.06_{(0.0156)}$	$417.14_{(0.125)}$	$208.57_{(0.0625)}$	$208.57_{(0.0625)}$	$208.57_{(0.0625)}$	$52.06_{(0.0156)}$
M21Q	$13.07_{(0.0039)}$	$209.41_{(0.0625)}$	$6.53_{(0.00195)}$[1]	$13.07_{(0.0039)}$[1]	$52.27_{(0.0156)}$	$26.13_{(0.0078)}$[1]
M21A	$102.94_{(0.0312)}$	$102.94_{(0.03120)}$	$25.69_{(0.0078)}$	$102.94_{(0.0312)}$[1]	$205.87_{(0.0625)}$	$25.69_{(0.0078)}$
M21F	$13.14_{(0.0039)}$[1]	$105.31_{(0.0312)}$[1]	$6.57_{(0.00195)}$[1]	$3.29_{(0.000975)}$[1]	$26.29_{(0.0078)}$[1]	$13.14_{(0.0039)}$[1]
M21Y	$13.20_{(0.0039)}$[1]	$211.60_{(0.0625)}$	$13.20_{(0.0039)}$[1]	$13.20_{(0.0039)}$[1]	$105.80_{(0.0312)}$	$6.60_{(0.00195)}$[1]
M21V	$25.91_{(0.0078)}$	$207.63_{(0.0625)}$	$25.91_{(0.0078)}$	$25.91_{(0.0078)}$	$103.81_{(0.0312)}$	$103.81_{(0.0312)}$
AAK	$101.59_{(0.0312)}$	$203.18_{(0.0625)}$	$50.71_{(0.0156)}$	$50.71_{(0.0156)}$	$50.71_{(0.0156)}$	$203.18_{(0.0625)}$
PAQ	$25.56_{(0.0078)}$	$204.81_{(0.0625)}$	$12.78_{(0.0039)}$[1]	$51.12_{(0.0156)}$	$102.40_{(0.0312)}$	$51.12_{(0.0156)}$
VGA	$100.25_{(0.0312)}$	$400.98_{(0.125)}$	$50.04_{(0.0156)}$	$400.98_{(0.125)}$	$400.98_{(0.125)}$	$50.04_{(0.0156)}$
PGA	$50.00_{(0.0156)}$	$400.63_{(0.125)}$	$25.00_{(0.0078)}$	$50.00_{(0.0156)}$	$200.31_{(0.0625)}$	$100.16_{(0.0312)}$
H27Q	$3.26_{(0.000975)}$[1]	$208.92_{(0.0625)}$	$3.26_{(0.000975)}$[1]	$3.26_{(0.000975)}$[1]	$52.15_{(0.0156)}$	$3.26_{(0.000975)}$[1]
S29D	$105.69_{(0.0312)}$	$211.38_{(0.0625)}$	$26.38_{(0.0078)}$	$26.38_{(0.0078)}$	$105.69_{(0.0312)}$	$105.69_{(0.0312)}$

[1]MIC value is 50% to 195% lower than that of wild type.

membrane (Breukink et al., 2003). The activity of nisin against gram-negative bacteria is diminished due to the presence of LPS in the outer membrane, which acts as a barrier and restricts the entry of the nisin peptide (Nikaido, 2003). Nisin, when used in combination with chelating agents such as EDTA (Stevens et al., 1991), in an environment of high salt concentration (Kuwano et al., 2005), with food-grade oils (Campion et al., 2017), or together with other hurdle technology systems, can be used to control gram-negative bacteria. Although Li et al. (2018) highlighted the merits of combining full or truncated nisin with pore-forming peptides to improve activity against gram-negative pathogens (Li et al., 2018), to date, only a few studies have identified bioengineered nisin variants that exhibit enhanced activity against gram-negative bacteria in isolation (Yuan et al., 2004; Field et al., 2012). Here we identified bioengineered nisin A variants with enhanced bactericidal activity against gram-negative *Thermus* strains. More specifically, we identified bioengineered nisin A variants of the N-terminal domain (K12V, M17Q, K12S, K12L, and K12Y), hinge region (N20S, M21F, M21Y, M21Q), and C-terminal domain (H27Q) that exhibited enhanced specific activity against *Thermus* strains. Moreover, M17Q and M21F showed enhanced antimicrobial activity against all *Thermus* strains tested and represent the first example of nisin peptides with aromatic residue that exhibit enhanced activity. Field et al. (2012) suggested that the increased activity of nisin derivatives against gram-negative bacteria was due to an increased ability of nisin to traverse the outer membrane (Field et al., 2012). This could be the case with *Thermus* also. It is also worth noting that *Thermus* contains exceptionally large porins, which might contribute to the sensitivity of the strains to nisin and bioengineered derivatives thereof (Nikaido, 2003). Other factors such as the lack of LPS but the presence of polar glycolipids and phosphoglycolipids with C_{16}, C_{18}, and C_{14} saturated and unsaturated fatty acids, is a feature of some strains of genus *Thermus* (Pask-Hughes and Williams, 1978; Silipo et al., 2004; Leone et al., 2006). This might also have a significant role in enhancing nisin activity, as LPS is thought to contribute to the nisin resistance of gram-negative bacteria (Nikaido, 2003). Further research is needed to identify the mechanism of action of nisin peptides against *Thermus*, and to clarify why certain derivatives possess greater potency than others.

Pink discoloration in cheese is a significant problem to cheese industries internationally, with associated pink bands under the surface or throughout the cheese potentially leading to product rejection by consumers. Semihard cheeses such as Emmental, Cheddar (noncolored), Romano, Gouda, and others are susceptible to this discoloration. Many reasons for this discoloration have been postulated by researchers, including an interaction among metabolites released by microbes present in cheese, high nitrates and tyrosine, photochemical oxidation, and the presence of pigments produced by

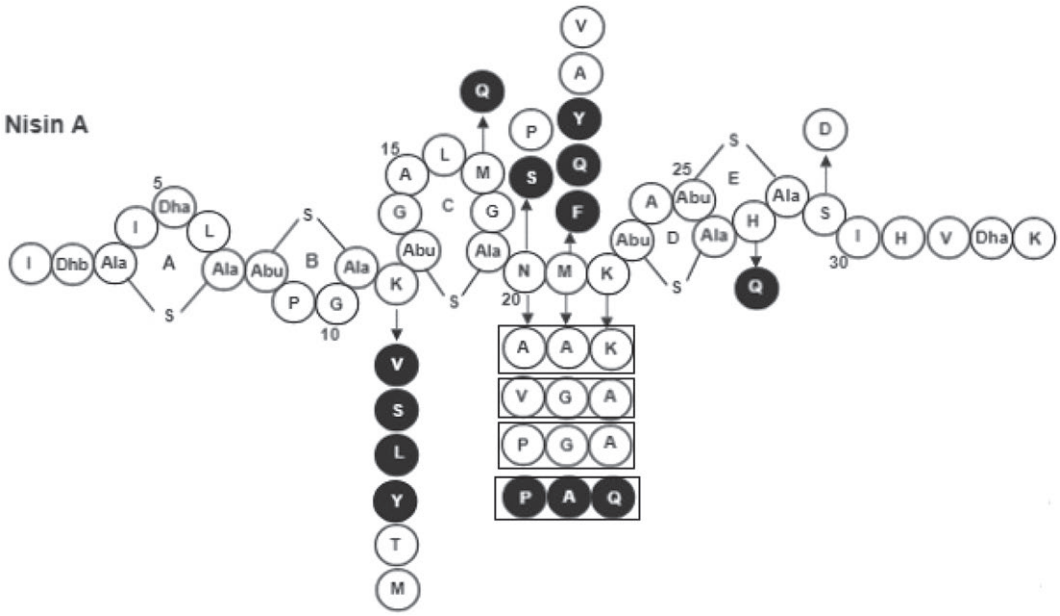

Figure 1. Nisin A peptide linear structure and bioengineered variants. Arrows denote the single and combination (joined circles in boxes) amino acid substitutions at certain positions. Black circles indicate enhanced specific activity of that bioengineered variant against certain *Thermus* strains (*Thermus thermophilus* HB27, *Thermus scotoductus* SE-1, *Thermus rehai*, *Thermus brockianus*, *Thermus oshimai*, and *Thermus aquaticus*). Dha = dehydroalanine; Dhb = dehydrobutyrine; Ala-S-Ala = lanthionine; Abu-S-Ala = beta-methyllanthionine.

cheese microbes. For more details on pink discoloration, the reader is directed to the review by Daly and colleagues on pink discoloration in commercial cheese (Daly et al., 2012). A recent metagenomic study within our group identified high levels of carotenoid-producing *T. thermophilus* in Swiss-type pink defective cheese. This species has not been regarded as being cheese-associated (Quigley et al., 2016). Ultimately, the current investigation to determine the ability of nisin and bioengineered variants thereof to inhibit the growth of *Thermus* bacteria could be a useful discovery for cheese industries. Nisin has been used as a biopreservative in dairy products such as cheese, and is known to inhibit such pathogens as *L. monocytogenes* and *Clostridium difficile* in cheese matrices (Davies et al., 1997; Sobrino-López and Martín-Belloso, 2008). A similar application of bioengineered nisin A variants to inhibit the growth of *Thermus* in cheese could possibly help in preventing pink defect caused by the presence of this bacterium, especially because strains that have been bioengineered through self-cloning to produce a bacteriocin with a single amino acid change are not regulated as genetically modified microorganisms (Field et al., 2018). The authors emphasize that the current study was based on studies in laboratory media, and the activity of nisin A and its derivatives might vary in a food system. We further note that nisin has some limitations, such as interactions with casein protein, lack of stability at pH 7, and partitioning in fat globules, which might affect its ability to exhibit proper specific activity in a dairy environment (Ibarra-Sánchez et al., 2020). Thus, further research will be needed to check the activity of these nisin peptides in cheeses' environment against *Thermus* and other certain bacteria and to understand the level of nisin required to exhibit its specific activity.

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