

Title	Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control Cronobacter sakazakii and Escherichia coli O157:H7
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Publication date	2017-02-07
Original Citation	Campion, A., Morrissey, R., Field, D., Cotter, P. D., Hill, C. and Ross, R. P. (2017) 'Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control Cronobacter sakazakii and Escherichia coli O157:H7', Food Microbiology, 65, pp. 254-263. doi:10.1016/j.fm.2017.01.020
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.fm.2017.01.020
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Download date	2026-10-12 08:57:20
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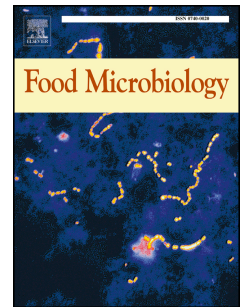
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Accepted Manuscript

Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control *Cronobacter sakazakii* and *Escherichia coli* O157:H7

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PII: S0740-0020(16)30065-X

DOI: [10.1016/j.fm.2017.01.020](https://doi.org/10.1016/j.fm.2017.01.020)

Reference: YFMIC 2729

To appear in: *Food Microbiology*

Please cite this article as: Alicia Campion, Ruth Morrissey, Des Field, Paul D. Cotter, Colin Hill, R. Paul Ross, Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control *Cronobacter sakazakii* and *Escherichia coli* O157:H7, *Food Microbiology* (2017), doi: 10.1016/j.fm.2017.01.020

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Highlights

- Bioengineered nisin variants and essential oils were tested for inactivation of C. sakazakii and E. coli O157:H7.
- Nisin variant and essential oil combinations caused extended lag phases of growth compared to nisin A-essential oil combinations.
- Nisin variant-carvacrol combinations significantly reduced C. sakazakii and E. coli O157:H7 compared to nisin A-carvacrol treatment.
- Nisin variant-carvacrol combinations caused complete inactivation of E. coli O157:H7 in apple juice compared to nisin A-carvacrol treatment.
- Commercial Nisaplin and citric acid combinations also resulted in complete inactivation of C. sakazakii in infant formula.

1 **Use of enhanced nisin derivatives in combination with food-**
2 **grade oils or citric acid to control *Cronobacter sakazakii* and**
3 ***Escherichia coli* O157:H7**

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21

22

Abstract

24

25 *Cronobacter sakazakii* and *Escherichia coli* O157:H7 are well known food-
26 borne pathogens that can cause severe disease. The identification of new
27 alternatives to heating to control these pathogens in foods, while reducing the
28 impact on organoleptic properties and nutritional value, is highly desirable. In this
29 study, nisin and its bioengineered variants, nisin V and nisin S29A, are used alone, or
30 in combination with plant essential oils (thymol, carvacrol and trans-
31 cinnamaldehyde) or citric acid, with a view to controlling *C. sakazakii* and *E. coli*
32 O157:H7 in laboratory-based assays and model food systems. The use of nisin
33 variants (30 μ M) with low concentrations of thymol (0.015%), carvacrol (0.03%) and
34 trans-cinnamaldehyde (0.035%) resulted in extended lag phases of growth compared
35 to those for corresponding nisin A-essential oil combinations. Furthermore, nisin
36 variants (60 μ M) used in combination with carvacrol (0.03%) significantly reduced
37 viable counts of *E. coli* O157:H7 (3-log) and *C. sakazakii* (4-log) compared to nisin A-
38 carvacrol treatment. Importantly, this increased effectiveness translated into food.
39 More specifically, sub-inhibitory concentrations of nisin variants and carvacrol
40 caused complete inactivation of *E. coli* O157:H7 in apple juice within 3 hours at room
41 temperature compared to that of the equivalent nisin A combination. Furthermore,
42 combinations of commercial Nisaplin and the food additive citric acid reduced *C.*
43 *sakazakii* numbers markedly in infant formula within the same 3 h period. These
44 results highlight the potential benefits of combining nisin and variants thereof with
45 carvacrol and/or citric acid for the inhibition of Gram negative food-borne
46 pathogens.

47

48 **Keywords**

49

50 *C. sakazakii*, *E. coli* O157:H7, Nisin, Essential oils, Apple juice, Infant formula milk

51

52 **1. Introduction**

53

54 *Cronobacter sakazakii* and *Escherichia coli* O157:H7 are both significant Gram
55 negative foodborne pathogens. They have garnered special notoriety because of
56 their association with life-threatening diseases. Their presence in food poses a
57 serious health risk for consumers and is a safety concern for the food industry.
58 Enterohaemorrhagic *E. coli* O157:H7 can cause devastating and severe illness such as
59 haemorrhagic colitis and haemolytic uremic syndrome. Approximately 10-15% of *E.*
60 *coli* O157:H7 infections result in haemolytic uremic syndrome, causing acute renal
61 failure in children and 3-5% of cases are fatal (Ho et al., 2013). There have been
62 several outbreaks associated with the consumption of food contaminated with *E. coli*
63 O157:H7 (Vidovic and Korber, 2014). Similarly, *C. sakazakii* can cause a range of
64 serious neonatal infections such as meningitis, septicaemia and enteritis (Drudy et
65 al., 2006; Gurtler et al., 2005). Several disease outbreaks have been associated with
66 the contamination of powdered infant formula milk (CDC, 2002; Iversen and
67 Forsythe, 2004). *C. sakazakii* has a high mortality rate of 40-80%, and death can
68 occur within hours (Bowen and Braden, 2006; Norberg et al., 2012). Infection may
69 also result in severe sequelae such as hydrocephalus, quadriplegia and retarded
70 neural development among survivors (Forsythe, 2005).

Heat treatments and chemical preservatives are commonly used as hurdles to control foodborne pathogens and spoilage bacteria. However, these processes may have undesirable effects, such as altering the nutritional and sensory properties of the food. Furthermore, there has been an increasing consumer demand for additive-free, minimally processed foods, while still maintaining adequate microbiological safety and stability. Therefore, the use of natural antimicrobials as food preservatives has been the focus of ever-increasing attention. Among these natural alternatives are bacteriocins. Bacteriocins are ribosomally synthesised, post-translationally modified peptides that are produced by bacteria and which are active against other bacteria. They can have a narrow range of activity within their own species or a broad spectrum of activity across genera (Cotter et al., 2005). Although there are numerous bacteriocins with food preservation potential, only nisin, produced by *Lactococcus lactis*, is used extensively. Nisin A has been assigned to the lantibiotic class of bacteriocins due to the presence of unusual amino acids that arise due to the post-translational modification of serine and threonine residues ultimately leading to the formation of lanthionine and β -methyllanthionine ring structures, respectively (Bierbaum and Sahl, 2009; Sahl et al., 1995). Nisin A is used in over 50 countries worldwide and has been approved for use by both the EU (E234) and the Food and Drug Administration (FDA) (Delves-Broughton, 1990). Nisin A functions through a unique dual mode of action. It binds to lipid II, an essential precursor to cell wall biosynthesis, while also inserting itself into the bacterial cell membrane. This facilitates pore formation and ultimately leads to the loss of solutes from the bacterial cell resulting in cell death (Wiedemann et al., 2004; Wiedemann et al., 2001).

95 The gene-encoded nature of nisin A allows for its manipulation in order to
96 modify its biological and physical properties. Indeed, recent research has shown that
97 bioengineering of nisin A can result in variants with greater potency towards food-
98 borne pathogens (Field et al., 2015b). One particular variant, M21V (nisin V), has
99 shown enhanced activity towards several Gram positive pathogens, including *Listeria*
100 *monocytogenes*, compared to that of nisin A (Field et al., 2010). Although nisin A is
101 effective against Gram positive bacteria such as *Staphylococci*, *Bacilli* and *Clostridia*
102 (Bierbaum and Sahl, 2009; Sobrino-López and Martín-Belloso, 2008), Gram negative
103 bacteria are generally not as sensitive. However, novel variants, such as nisin S29A
104 and S29G, with enhanced activity towards Gram negative food-associated pathogens
105 exist (Field et al., 2012). Nisin A may also be effective against Gram negatives if their
106 outer membrane is destabilized with chelating agents (Stevens et al., 1991).
107 Membrane disruption/permeabilisation is also thought to be the basis for the
108 observation that nisin, when combined with the phenolic compounds carvacrol and
109 thymol which possess membrane permeability properties, exhibit enhanced activity
110 against Gram negative bacteria by permitting nisin to pass through the protective
111 outer membrane (Helander et al., 1998). In fact, there are several studies
112 demonstrating that nisin and essential oil combinations exhibit enhanced inhibitory
113 effects against both Gram positive and Gram negative bacteria (Ettayebi et al., 2000;
114 Olasupo et al., 2003; Olasupo et al., 2004; Periago and Moezelaar, 2001; Pol and
115 Smid, 1999; Yuste and Fung, 2004). More recently, nisin-containing semi-purified
116 preparations in combination with carvacrol and trans-cinnamaldehyde were
117 established to more effectively inhibit *L. monocytogenes* than either treatment alone
118 (Field et al., 2015a).

The aim of this study was to evaluate the antimicrobial activity of nisin A, or the bioengineered nisin derivatives nisin V and S29A, when combined with the essential oils, thymol, carvacrol or trans-cinnamaldehyde, or citric acid against the Gram negative pathogens *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} or *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

The nisin producing strains and *lux*-tagged bacterial strains used in this study are listed in Table 1. *L. lactis* strains were grown in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) or GM17 agar at 30°C. *E. coli* and *C. sakazakii* cultures were grown in Luria-Bertani (LB) broth or agar at 37°C. When required, antibiotics were used where indicated at the following concentrations: chloramphenicol at 10 µg ml⁻¹ for *L. lactis* and erythromycin at 500 µg ml⁻¹ for *E. coli* and *C. sakazakii*. Stock solutions of thymol (Sigma) were prepared at 50 mg ml⁻¹ in 50% ethanol and stored

at -20°C. Carvacrol and trans-cinnamaldehyde (Sigma) were diluted from stock (0.976 g ml⁻¹ and 1.05 g ml⁻¹, respectively) in 50% ethanol to the desired concentration. Stock solutions of Nisaplin (Sigma) and citric acid (Sigma) were prepared at 100 mg ml⁻¹ and 500 mM in sdH₂O, respectively, filter sterilised and diluted to the desired concentration. In all experiments, the concentration of ethanol did not exceed 2% (vol/vol).

2.2 Nisin purification

Purification of wild type nisin A and nisin derivatives were carried out as described previously (Field et al., 2010). Briefly, overnight cultures of nisin producing strains 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 cell pellet was resuspended in 300ml of 70% isopropanol 0.1% TFA and magnetically stirred for 3 h at room temperature. Cell debris was removed by centrifugation 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 isopropanol was evaporated off using a rotary evaporator (Buchi) to a volume of 160ml and the sample pH adjusted to approximately 4.2. The sample was applied to a 10g (60ml) 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⁻¹. Fractions containing nisin A and nisin derivative peptides were collected and subjected to Mass Spectrometry with a Shimadzu Biotech MALDI-TOF Mass Spectrometer (AXIMA-CFR plus model).

2.3 Mass spectrometry

For colony mass spectrometry analysis, bacteria were collected with sterile plastic loops and mixed with 50 µl of 70% isopropanol adjusted to pH2 with HCl. The suspension was vortexed, centrifuged at 14,000 r.p.m. for 2 minutes and the supernatant retained for analysis. Mass spectrometry in all cases was performed with an Axima CFR plus matrix-assisted laser desorption/ionisation time-of-flight (MALDI TOF) mass spectrometer (Shimadzu Biotech, Manchester, UK.) A 0.5 µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10mg ml⁻¹ in 50% acetonitrile-0.1% (v/v) trifluoroacetic acid) was placed onto the target and left for 1-2 minutes before being removed. The residual solution was then air dried and the sample solution (re-suspended lyophilized powder or colony mass spectrometry supernatant) was positioned onto the pre-coated sample spot. Matrix solution (0.5 µl) was added to the sample and allowed to air dry. The sample was subsequently analysed in positive-ion reflectron mode.

2.4 Minimum inhibitory concentration (MIC) assays

The MIC of nisin peptides against Gram negative organisms were carried out in triplicate in microtitre plates as previously described (Field et al., 2010). Briefly, 96-well microtitre plates were pre-treated with phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) and subsequently incubated at 37°C for 30 minutes. Wells were washed with PBS and allowed to air-dry before the addition of 100 µl double-strength LB broth. Gram negative strains were grown overnight in LB broth at 37°C, subcultured into fresh LB broth and grown to mid-logarithmic phase ($OD_{600nm} \sim 0.5$). The cells were harvested by centrifugation, washed with 10 mM SPB at pH 7.4 and diluted to 1×10^5 cfu ml⁻¹ in 10 mM SPB pH 7.4. Nisin and nisin derivative purified peptides were resuspended in double-strength LB broth to a stock concentration of 60 µM. Peptides were then adjusted to a starting concentration of 15 µM and 2-fold dilutions of the nisin peptides were carried out in the 96-well plates. Target organisms were added and plates were incubated at room temperature overnight (~16 h). The MIC was taken as the lowest concentration of peptide at which growth was inhibited. The MIC of essential oils against Gram negative strains were carried out as above but with minor variations; BSA treatment of 96-well plates were not required and essential oils were diluted to a starting concentration of 2 mg ml⁻¹ for serial dilution of thymol, carvacrol and trans-cinnamaldehyde.

2.5 Growth/Kill assays

Growth and kill assays were carried out using representative strains as a consequence of the limited amount of pure material available. For growth assays, overnight cultures of target strains were harvested by centrifugation, washed in 10 mM SPB pH 7.4 and transferred (1×10^7 cfu ml⁻¹ in a 1.0 ml volume) into LB broth containing nisin purified peptide alone and in combination with one of the essential oils being investigated. A volume of 200 µl was transferred to a 96-well plate (Genetix) and cell growth was measured spectrophotometrically using a Spectra Max 340 spectrophotometer (Molecular Devices, Sunnyvale, California) for 24 h. For kill assays, overnight cultures of target strains were again harvested by centrifugation, washed in 10 mM SPB pH 7.4 and transferred (1×10^7 cfu ml⁻¹ in a 0.5 ml volume) into LB broth containing nisin purified peptide alone and in combination with one of the essential oils. Samples were incubated for 3 h at room temperature before serial dilution and enumeration on LB agar plates. All experiments were carried out in triplicate.

2.6 Infant milk formula trial

Commercially available powdered infant formula (PIF) (Aptamil™ First Milk) was prepared according to manufacturer's instructions and brought to room temperature. The pH of the reconstituted PIF was 6.8 as determined with a pH meter. An overnight culture of *C. sakazakii* NCTC 8155::p16*Slux*-P_{help} was washed in 10 mM SPB pH 7.4, diluted and inoculated into reconstituted PIF at a final

concentration of 1×10^5 cfu ml⁻¹. PIF samples were treated with 201.12 µg ml⁻¹ (60 µM) of nisin A, nisin V or nisin S29A, alone and in combination with carvacrol at a concentration of 300 µg ml⁻¹. Samples with carvacrol alone, *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} alone and PIF alone served as controls. Samples were incubated at room temperature and *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} levels were determined through serial dilution and plate count technique on Druggan-Forsythe-Iversen (DFI) agar at 3 h. Where Nisaplin (Sigma), and citric acid (Sigma) were employed, concentrations of 10 mg ml⁻¹ and 30 mM were used, respectively. Nisaplin (containing 2.5% nisin) was resuspended in sdH₂O and filter sterilised before use. The addition of citric acid did not significantly alter the pH of PIF. All experiments were carried out in triplicate.

2.7 Apple juice trial

Commercially available apple juice was brought to room temperature, filtered and the pH determined as 3.2 with a pH meter. An overnight culture of *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} was washed in 10 mM SPB pH 7.4, diluted and inoculated into the apple juice at a final concentration of 1×10^5 cfu ml⁻¹. Apple juice samples were treated with 100.56 µg ml⁻¹ (30µM) of nisin A, nisin V or nisin S29A, alone and in combination with carvacrol at a concentration of 75 µg ml⁻¹. Samples with carvacrol alone, *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} alone and apple juice alone served as controls. Samples were incubated at room temperature and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} levels were determined through serial dilution and

plate count technique on Sorbitol MacConkey agar at 3 h. All experiments were carried out in triplicate.

2.8 Statistical analysis

CFU data was transformed to $\log_{10}$ prior to analysis using the statistical software package GraphPad Prism 6. All comparisons were based on the mean $\pm$ standard deviation. Statistical significance was determined via GraphPad prism t-test. In all cases, a P value less than 0.05 were considered to be statistically significant.

Parametric data was analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Non-parametric data was analysed using the Kruskal-Wallis one way ANOVA followed by Dunn's multiple comparisons test. Asterisks rating of *, **, *** or **** indicates statistically significant differences between groups ($P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, respectively).

3. Results

3.1 Minimum inhibitory concentration assays

Nisin V and nisin S29A are previously generated nisin derivatives that exhibit enhanced activity against a number of targets arising from single amino acid alterations (Fig. 1) (Field et al., 2012). These peptides and nisin A were purified and, following purification and freeze-drying, mass spectrometry analysis was performed.

286 Peptide masses of 3,321 Da, 3,336 Da and 3,353 Da were obtained for nisin peptides
 287 V, S29A and nisin A as expected (data not shown). To more accurately quantify the
 288 specific activity of the peptides, broth-based MIC determination assays were carried
 289 out using purified peptides against the chosen Gram negative targets (Table 2). Nisin
 290 S29A exhibited two-fold greater specific activity than nisin A against *E. coli* O157:H7
 291 TUV 93-0::p16S*lux*-P_{help} (3.75 μ M and 7.5 μ M, respectively) and *C. sakazakii* NCTC
 292 8155::p16S*lux*-P_{help} (1.87 μ M and 3.75 μ M, respectively). In the case of nisin V,
 293 enhanced specific activity compared to nisin A was observed for *E. coli* O157:H7 TUV
 294 93-0::p16S*lux*-P_{help} (3.75 μ M and 7.5 μ M, respectively) but activity was equal to that
 295 of nisin A when examined against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help}. These
 296 results demonstrate for the first time the enhanced activity of nisin V against a
 297 Gram-negative strain (*E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}) in a broth-based MIC
 298 assay. The enhanced efficacy of S29A compared to nisin A against some Gram
 299 negative strains has previously been reported (Field et al., 2012).

300 The susceptibility of the Gram-negative strains to the essential oils thymol,
 301 carvacrol and trans-cinnamaldehyde was also assessed in order to ascertain
 302 appropriate concentrations for combinatorial assays (Table 2). The essential oils
 303 were found to be inhibitory at a concentration of 250 μ g ml⁻¹ against *E. coli* O157:H7
 304 TUV 93-0::p16S*lux*-P_{help} and *C. sakazakii* NCTC 8155::p16S*lux*-P_{help}, with the
 305 exception that thymol prevented the growth of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help}
 306 at a concentration of 125 μ g ml⁻¹. These values are consistent with those previously
 307 reported (Hylgaard et al., 2012).

308

309 3.2 Growth and kill-curve assays

310

311 Having shown the increased specific activity of the nisin variants against *C.*
 312 *sakazakii* NCTC 8155::p16*Slux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16*Slux*-P_{help}, a
 313 more detailed investigation of the impact of the nisin peptides alone, and in
 314 combination with essential oils, on bacterial growth was examined. Due to relatively
 315 large inoculum (10^7 cfu ml⁻¹) employed for growth curve analysis, the impact of
 316 concentrations of 30 μ M and 15 μ M nisin peptides against *C. sakazakii* NCTC
 317 8155::p16*Slux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16*Slux*-P_{help}, respectively, was
 318 tested along with varying concentrations (75-200 μ g ml⁻¹) of essential oils. When
 319 nisin V was used in combination with 100 μ g ml⁻¹ thymol and 125 μ g ml⁻¹ carvacrol or
 320 trans-cinnamaldehyde, a more profound delay in growth was observed compared to
 321 that of either treatment alone against *C. sakazakii* NCTC 8155::p16*Slux*-P_{help} (Figs. 2).
 322 Nisin S29A-essential oil combinations were also better than their nisin A-essential oil
 323 equivalents (Fig. 2). However, statistically significant differences in bacterial
 324 inhibition were recorded only when nisin variants (V and S29A) were used in
 325 combination with the essential oil carvacrol and trans-cinnamaldehyde (Figs. 2C; P-
 326 value = 0.0209, 2E; P-value = 0.0007 and 2F; P-value = 0.0014), as compared to the
 327 nisin variant used alone. No significant difference in bacterial inhibition was
 328 observed for nisin variants in combination with thymol (Fig. 2A; P-value = 0.0681 and
 329 2B; P-value = 0.5645). Growth curves with *E. coli* O157:H7 TUV 93-0::p16*Slux*-P_{help}
 330 show that combinations of nisin V and thymol (100 μ g ml⁻¹) or carvacrol (200 μ g ml⁻¹)
 331 result in a longer lag phase than when either treatment is used singly (Figs. 3A and
 332 3C). A similar pattern was observed when nisin S29A was used in combination with
 333 thymol or carvacrol (Figs. 3B and 3D). Indeed, statistically significant differences in

334 bacterial inhibition were recorded when nisin variants (V and S29A) used in
 335 combination with the essential oils thymol and carvacrol (Figs. 3A; P-value =0.0022,
 336 3B; P-value = 0.0176, 3C; P-value =0.0029 and 3D; P-value = 0.0001), as compared to
 337 the nisin variant used alone. No significant difference in lag time was observed for
 338 nisin variants in combination with 75 $\mu\text{g ml}^{-1}$ trans-cinnamaldehyde (Fig. 3E; P-value
 339 =0.9675 and 3F; P-value =0.4427). Ultimately, the most significant delay in growth
 340 was observed when nisin V and carvacrol were combined against *E. coli* O157:H7
 341 TUV 93-0::p16S*lux*-P_{help} (Fig. 3C). Overall, it was apparent that, in general, the use of
 342 nisin V or nisin S29A resulted in greater inhibitory effects on growth than was
 343 observed when nisin A alone was used, and this phenomenon was also apparent
 344 when the bacteriocins were used together with essential oils.

345 Following on from this, the bactericidal activities of nisin peptides and
 346 essential oils against the Gram negative pathogens were investigated through kill
 347 curve analysis. *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-
 348 0::p16S*lux*-P_{help} were treated respectively with 60 μM and 30 μM of each nisin
 349 peptide in combination with 150 $\mu\text{g ml}^{-1}$ thymol, 300 $\mu\text{g ml}^{-1}$ carvacrol or 350 $\mu\text{g ml}^{-1}$
 350 trans-cinnamaldehyde (Figs. 4 and 5). In general, an approximate 1-log reduction in
 351 pathogen cell numbers were observed when either nisin peptides or essential oils
 352 were used alone, with the exception that carvacrol and trans-cinnamaldehyde had
 353 no effect against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (Figs. 4B and 4C, respectively)
 354 and the use of thymol alone against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} resulted
 355 in an approximate 3.5-log reduction in cell numbers (Fig. 5A). The killing effect of
 356 nisin peptides was more marked by the addition of essential oils. The antimicrobial
 357 activity of nisin peptides was significantly enhanced when used in combination with

thymol (~2-log reduction), carvacrol (~4-log reduction) and trans-cinnamaldehyde (~1.5-log reduction) against *C. sakazakii* NCTC 8155::p16Slux-P_{help} (Fig. 4). A similar significant level of inactivation of *E. coli* O157:H7 TUV 93-0::p16Slux-P_{help} was achieved when thymol (~4.5-log reduction), carvacrol (~3-log reduction) and trans-cinnamaldehyde (~2-log reduction) were used in combination with nisin peptides (Fig. 5). It was also established that the use of bioengineered nisin derivatives in combination with essential oils was more effective than nisin A-essential oil combinations. A significantly greater reduction in *E. coli* O157:H7 TUV 93-0::p16Slux-P_{help} was observed when thymol was used in combination with nisin V or nisin S29A, rather than nisin A ($P < 0.05$) (Fig. 5A). A similar observation was made when trans-cinnamaldehyde and *C. sakazakii* NCTC 8155::p16Slux-P_{help} were utilised, in that a considerable reduction in viable cell numbers was observed when the essential oil was used in conjunction with nisin V (Fig. 4C). Furthermore, an increased bactericidal activity was seen when combinations of nisin V and nisin S29A with carvacrol were used, resulting in a 2-log greater reduction in *C. sakazakii* NCTC 8155::p16Slux-P_{help} cell counts than was the case when a nisin A-carvacrol combination was used (Fig. 4B).

3.4 Model food trials

Having shown the enhanced potency of bioengineered nisin variants in combination with carvacrol against both Gram negative pathogens using kill curve assays, this essential oil was selected for further investigation in model food systems. To this end, commercially available powdered infant formula milk and commercially

382 produced apple juice were chosen as they have been associated with disease
 383 outbreaks of *C. sakazakii* and *E. coli* O157:H7, respectively. For the powdered infant
 384 formula milk studies, nisin A, nisin V or nisin S29A (60 μ M) were added alone or in
 385 combination with carvacrol (300 μ g ml⁻¹) (Fig. 6A). The addition of carvacrol did not
 386 significantly alter the pH. The milk was subsequently spiked with *C. sakazakii* NCTC
 387 8155::p16S*lux*-P_{help} at a concentration of 1×10^5 cfu ml⁻¹ and incubated at room
 388 temperature for 3 h. Serial dilution and subsequent plate counts revealed that nisin
 389 and/or essential oil treatment had no effect on *C. sakazakii* NCTC 8155::p16S*lux*-
 390 P_{help}, as cell numbers remained unaltered (Fig 6A). It was noted, however, that the
 391 addition of the food-grade antimicrobial, citric acid (30 mM) reduced viable cell
 392 numbers of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (~0.5-log reduction) while
 393 combinations of nisin and citric acid resulted in a significant decrease of *C. sakazakii*
 394 NCTC 8155::p16S*lux*-P_{help} cell numbers (1-log reduction) compared to nisin usage
 395 alone (P<0.001) (Fig. 6B). Combinations of all three antimicrobials improved
 396 antibacterial activity relative to nisin and the essential oil carvacrol. While there was
 397 a numerical improvement in activity relative to that of citric acid, this was not
 398 significant (Fig. 6C). Additionally, nisin variant combinations did not display an
 399 enhanced potency compared to the equivalent nisin A combined treatment.

400 Given that the essential oil/nisin combinations did not significantly enhance
 401 the efficacy of citric acid against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} in infant
 402 formula, we investigated the possibility that another nisin-based, food grade
 403 formulation could prove to be effective. For this purpose Nisaplin, a commercial
 404 formulation containing nisin A was used together with citric acid. Notably, this

combination brought about a significant >3-log reduction in bacterial cell counts (Fig. 7). This effect was not further augmented through the addition of carvacrol.

The commercially produced apple juice was filtered and the pH measured. Nisin A, nisin V or nisin S29A (30 μM) was added to the juice alone or in combination with low level of carvacrol (75 $\mu\text{g ml}^{-1}$) before *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} was introduced at a concentration of 1×10^5 cfu ml^{-1} . Following incubation for 3 h at room temperature, serial dilutions and plate counts on Sorbitol MacConkey agar were carried out to enumerate bacterial cell counts. When nisin A, nisin V, nisin S29A or carvacrol were used alone, an approximate 1-log reduction in *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} cell numbers were obtained. The effectiveness of nisin A was significantly improved when used in conjunction with carvacrol, resulting in a 2.5-log reduction in cells counts compared to that of the initial inoculum. Notably, the combinations of nisin V and nisin S29A with carvacrol were even more effective in that a 3-log reduction in bacterial cell counts was attained over the 3 h period (Fig. 8). More importantly, these results demonstrate that the enhanced effectiveness of nisin variants observed using laboratory-based assays are retained and can be translated to food systems.

4. Discussion

It has previously been reported that the phenolic compounds carvacrol and thymol have the ability to degrade the outer membranes of the Gram negative bacteria, *E. coli* and *Salmonella Typhimurium* (Helander et al., 1998). This phenomenon likely explains why exposure to these compounds increases the

sensitivity of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} to nisin. Our investigations also highlight the enhanced potency of nisin when combined with trans-cinnamaldehyde. In the latter case, the mechanism involved may be different as trans-cinnamaldehyde does not disintegrate the outer membrane like carvacrol and thymol, but is believed that at sub-inhibitory concentrations, inhibits the activity of trans-membrane ATPase (Gill and Holley, 2006a, b).

This study also demonstrates the frequently superior activity of bioengineered nisin variants over their wild type nisin A equivalent when used in combination with a variety of essential oils. In growth curve assays, nisin V-essential oil and nisin S29A-essential oil combinations, except when combined with trans-cinnamaldehyde against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}, surpassed the activity of their nisin A counterparts as observed by the longer delay in growth. With respect to time-kill assays, nisin variant-combinations outperformed their corresponding nisin A-combinations by at least 1-log reduction in cell numbers against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (carvacrol and trans-cinnamaldehyde) and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} (thymol and carvacrol). This increased effectiveness did not translate into powdered infant formula milk (Fig. 6A). It is known that the greater availability of nutrients in foods compared to laboratory media may enable bacterial cells to repair damaged cells faster (Gill et al., 2002). As a consequence of this protective nature, a greater concentration (approximately two-fold) of essential oils are required to achieve the same effect in foods, such as semi-skimmed milk (Karatzas et al., 2001). Instead of increasing concentrations of carvacrol and potentially altering the sensory properties of the infant milk formula,

the preservative citric acid (30 mM) was incorporated. A significant reduction (1-log) in bacterial counts was observed when nisin and citric acid were used simultaneously compared to nisin alone (Fig. 6B). However, the combination of all three antimicrobial agents did not improve significantly on the activity of citric acid against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (Fig. 6C). The substitution of nisin peptides for food-grade Nisaplin (10 mg ml⁻¹) proved most effective as >3-log reduction in *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} cell counts was observed after treatment with Nisaplin and citric acid (Fig. 7). Nisaplin contains 2.5% active nisin and the remainder is made up of a balance of sodium chloride and denatured milk solids. The high percentage of salts present is likely to contribute to antimicrobial activity. The nisin-containing formulation was employed as it is commercially available and approved for use in over 50 countries worldwide. Should equivalent forms of nisin V/S29A be generated, there is the potential for these peptides to be used in the same way with the possibility of even greater antimicrobial efficacy.

Due to the demand for minimally processed foods, researchers have previously investigated the use of natural antimicrobials including nisin and cinnamon in apple juice (Yuste and Fung, 2004). In this study, we also investigated the merits of using nisin in combination with the essential oil carvacrol in apple juice. The combination of the bioengineered nisin variants with carvacrol accelerated bacterial death, resulting in a >3-log reduction in *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} cell counts after 3 h (Fig. 8). The low pH of the apple juice may partially contribute to this effect. It is also known that essential oils are most effective at acidic pHs (Burt, 2004) due to the increase in hydrophobicity and better diffusion into the lipid phase of the membrane (Juven et al., 1994). Moreover, nisin is more

stable at acidic pH and therefore more effective (Delves-Broughton, 1990). While, the manufacture of fruit juices should include effective treatments so as to result in a cumulative 5-log reduction in the numbers of *E. coli* O157:H7 as specified by the Food and Drug Administration's Guidance for Industry (FDA, 2001), the detection limit in this study was 10^2 cfu ml⁻¹ and thus the use of more sensitive detection methods may reveal that this cumulative reduction can be achieved.

The use of essential oils in foods has been limited due to the high concentrations required to achieve sufficient antimicrobial activity (Hyldgaard et al., 2012). Notably, however, this study shows that relatively low concentrations of carvacrol (approximately 0.0075%) could be used in combination with nisin to inhibit *E. coli* O157:H7 TUV 93-0::p16*Slux*-P_{help} in apple juice. Before commercial application, sensory studies would have to be carried out to determine the organoleptic properties of apple juice with essential oils. Although, upon addition of such a low concentration of carvacrol in this study, no adverse aroma was observed. Recently, sensory evaluations were carried out to assess the consequence of adding 75 µl L⁻¹ (~63.75 µg ml⁻¹) of lemon essential oil to apple juice. Researchers found that this relatively small concentration of essential oil did not decrease the acceptability of the sample or the organoleptic properties (Espina et al., 2012). Moreover, all of the tested essential oils have GRAS status by both the EU and the FDA (EU, 2012; FDA, Revised 2014a, b) meaning that their addition could be permitted if they were used in the minimum quantities and with good manufacturing processes.

5. Conclusion

The combination of nisin peptides and essential oils could pave the way for new hurdle concepts when it comes to food preservation, in particular towards Gram negative bacteria. Moreover, the combinatory effects could lead to reduced treatment intensity and/or antimicrobial dosage and therefore avoid undesirable sensory and nutritional properties in food. Such combinations could enhance food safety, shelf life and quality while also meeting consumer demands for more natural, preservative-free foods.

Acknowledgements

This work was carried out through a PhD Programme in Molecular Cell Biology funded by the Programme for Research in Third-Level Institutions (PRTL) awarded to AC. Work in the authors' laboratory is supported by the Irish Government under the National Development Plan, through Science Foundation Ireland Investigator awards (10/ IN.1/B3027) and (06/IN.1/B98) (<http://www.sfi.ie>). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank Paula O'Connor for mass spectrometry and Paddy O'Reilly for technical assistance.

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Tables

Strains/Lux-tagged bacteria	Relevant characteristics or source of strains	Reference
<i>Lactococcus lactis</i> NZ9700	Wild-type nisin producer	(Kuipers et al., 1993; Kuipers et al., 1998)
<i>Lactococcus lactis</i> NZ9800	<i>L. lactis</i> NZ9700ΔnisA	(Kuipers et al., 1993; Kuipers et al., 1998)
<i>Lactococcus lactis</i> NZ9800 pCI372nisA	Wild-type nisin-producing strain	(Field et al., 2008)
<i>Lactococcus lactis</i> NZ9800 pCI372nisA::M21V	Wild-type producing strain + alteration at position 21	(Field et al., 2010)
<i>Lactococcus lactis</i> NZ9800 pCI372nisA::S29A	Wild-type nisin-producing strain + alteration at position 29	(Field et al., 2012)
<i>Cronobacter sakazakii</i> NCTC 8155::p16Slux-P _{help}	Isolated from dried milk powder and transformed with p16Slux-P _{help} plasmid	UCC Culture Collection
<i>Escherichia coli</i> O157:H7 TUV 93-0::p16Slux-P _{help}	Derived from strain EDL933 and transformed with p16Slux-P _{help}	UCC Culture Collection

Table 1

List of nisin-producing strains and *lux*-tagged bacterial strains used in this study,
including relevant characteristics and references.

Indicator organism	Nisin A $\mu\text{g ml}^{-1}$ (μM)	Nisin V $\mu\text{g ml}^{-1}$ (μM)	Nisin S29A $\mu\text{g ml}^{-1}$ (μM)	Thymol $\mu\text{g ml}^{-1}$	Carvacrol $\mu\text{g ml}^{-1}$	Cinnamaldehyde $\mu\text{g ml}^{-1}$
<i>C. sakazakii</i> NCTC 8155::p16S <i>lux</i> -P _{help}	12.57 (3.75)	12.57 (3.75)	6.28 (1.875) (P<0.001)	125	250	250
<i>E. coli</i> O157:H7 TUV 93-0::p16S <i>lux</i> -P _{help}	25.14 (7.5)	12.57 (3.75) (P<0.001)	12.57 (3.75) (P<0.001)	250	250	250

Table 2

Minimum inhibitory concentration determinations of nisin A, nisin V, nisin S29A and the essential oils thymol, carvacrol and trans-cinnamaldehyde against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}. Results are expressed as the mean of triplicate assays. Values in bold represent statistical difference between nisin variants and wild type nisin A (Student's t-test: P<0.05).

Figure legends

Fig. 1. Structure and mass spectrometry analysis of nisin A and its derivatives. **(A)**

Structural composition of nisin A. Blue circles indicate where amino acids were altered resulting in the generation of nisin variants with enhanced activity.

Figure 2. Growth curve analysis of *C. sakazakii* NCTC8155::p16*Slux*-P_{help} with 30μM

nisin A (open square), **(A)** nisin V (open diamond), 100μg/ml thymol (open circle) and combinations of nisin A and thymol (closed square), nisin V and thymol (closed

diamond) **(B)** nisin S29A (open inverted triangle), 100μg/ml thymol (open circle) and

combinations of nisin A and thymol (closed square), nisin S29A and thymol (closed

747 inverted triangle). **(C)** nisin V (open diamond), 125µg/ml carvacrol (open circle) and
 748 combinations of nisin A and carvacrol (closed square), nisin V and carvacrol (closed
 749 diamond) **(D)** nisin V (open diamond), nisin S29A (open inverted triangle), 125µg/ml
 750 carvacrol (open circle) and combinations of nisin A and carvacrol (closed square) and
 751 nisin S29A and thymol (closed inverted triangle). **(E)** nisin V (open diamond),
 752 125µg/ml trans-cinnamaldehyde (open circle) and combinations of nisin A and
 753 trans-cinnamaldehyde (closed square), nisin V and trans-cinnamaldehyde (closed
 754 diamond) **(F)** nisin S29A (open inverted triangle), 125µg/ml trans-cinnamaldehyde
 755 (open circle) and combinations of nisin A and trans-cinnamaldehyde (closed
 756 square) and nisin S29A and trans-cinnamaldehyde (closed inverted triangle).

757

758 **Figure 3.** Growth curve analysis of *E. coli* O157:H7 TUV93-0::p16S*lux*-P_{help} with 30µM
 759 nisin A (open square), **(A)** nisin V (open diamond), 100µg/ml thymol (open circle) and
 760 combinations of nisin A and thymol (closed square), nisin V and thymol (closed
 761 diamond) **(B)** nisin S29A (open inverted triangle), 100µg/ml thymol (open circle) and
 762 combinations of nisin A and thymol (closed square), nisin S29A and thymol (closed
 763 inverted triangle). **(C)** nisin V (open diamond), 125µg/ml carvacrol (open circle) and
 764 combinations of nisin A and carvacrol (closed square), nisin V and carvacrol (closed
 765 diamond) **(D)** nisin V (open diamond), nisin S29A (open inverted triangle), 125µg/ml
 766 carvacrol (open circle) and combinations of nisin A and carvacrol (closed square) and
 767 nisin S29A and thymol (closed inverted triangle). **(E)** nisin V (open diamond),
 768 125µg/ml trans-cinnamaldehyde (open circle) and combinations of nisin A and
 769 trans-cinnamaldehyde (closed square), nisin V and trans-cinnamaldehyde (closed
 770 diamond) **(F)** nisin S29A (open inverted triangle), 125µg/ml trans-cinnamaldehyde

(open circle) and combinations of nisin A and trans-cinnamaldehyde (closed square) and nisin S29A and trans-cinnamaldehyde (closed inverted triangle).

Fig. 4. Kill effect of nisin A derivatives in combination with essential oils against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help}. Kill curve analysis of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} with 60 µM of each peptide both alone and in combination with (A) 150 µg ml⁻¹ thymol (THY), (B) 300 µg ml⁻¹ carvacrol (CA) and (C) 350 µg ml⁻¹ trans-cinnamaldehyde (CN) in LB broth after 3 hours at room temperature.

Fig. 5. Kill effect of nisin A derivatives in combination with essential oils against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}. Kill curve analysis of *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} with 30 µM of each nisin peptide both alone and in combination with (A) 150 µg ml⁻¹ thymol (THY), (B) 300 µg ml⁻¹ carvacrol (CA) and (C) 350 µg ml⁻¹ trans-cinnamaldehyde (CN) in LB broth after 3 hours at room temperature. ND, not detected.

Fig. 6. Combinations of nisin derivatives, carvacrol and citric acid against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula. Model food analysis of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula with 60 µM of each nisin peptide both alone and in combination with (A) 300 µg ml⁻¹ carvacrol (CA), (B) 30 mM citric acid (CT) and (C) 300 µg ml⁻¹ carvacrol and 30 mM citric acid after 3 hours at room temperature.

Fig. 7. Combinations of commercial Nisaplin, carvacrol and citric acid against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula. Model food analysis of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula with 10 mg ml⁻¹ of commercial Nisaplin (Sigma) containing 2.5% nisin both alone and in combination with 300 µg ml⁻¹ carvacrol (CA) and 30 mM citric acid (CT) after 3 hours at room temperature. ND, not detected (detection limit of 10² cfu ml⁻¹).

Fig. 8. Combinations of nisin A derivatives and carvacrol against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} in apple juice. Model food analysis of *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} in apple juice with 30 µM of each nisin peptide both alone and in combination with 75 µg ml⁻¹ carvacrol (CA) after 3 hours at room temperature. ND, not detected (detection limit of 10² cfu ml⁻¹).

