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# 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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ABSTRACT

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

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## 1. Introduction

*Cronobacter sakazakii* and *Escherichia coli* O157:H7 are both significant Gram negative foodborne pathogens. They have garnered special notoriety because of their association with life-threatening diseases. Their presence in food poses a serious health risk for consumers and is a safety concern for the food industry. Enterohaemorrhagic *E. coli* O157:H7 can cause devastating and severe illness such as haemorrhagic colitis and haemolytic uremic syndrome. Approximately 10–15% of *E. coli* O157:H7 infections result in haemolytic uremic syndrome, causing acute renal failure in children and 3–5% of cases are fatal (Ho et al., 2013). There have been several outbreaks associated with the consumption of food contaminated with *E. coli* O157:H7 (Vidovic and Korber, 2016). Similarly, *C. sakazakii* can cause a range of serious neonatal infections such as meningitis, septicaemia and enteritis (Drudy et al., 2006; Gurtler et al., 2005). Several disease outbreaks have been associated with the contamination of powdered infant formula milk (CDC, 2002; Iversen and Forsythe, 2004). *C. sakazakii* has a high mortality rate of 40–80%, and death can occur within hours (Bowen and Braden, 2006; Norberg et al., 2012). Infection may also result in severe sequelae such as hydrocephalus, quadriplegia and

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retarded 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., 2001, 2004).

The gene-encoded nature of nisin A allows for its manipulation in order to modify its biological and physical properties. Indeed, recent research has shown that bioengineering of nisin A can result in variants with greater potency towards food-borne pathogens (Field et al., 2015b). One particular variant, M21V (nisin V), has shown enhanced activity towards several Gram positive pathogens, including *Listeria monocytogenes*, compared to that of nisin A (Field et al., 2010). Although nisin A is effective against Gram positive bacteria such as *Staphylococci, Bacilli* and *Clostridia* (Bierbaum and Sahl, 2009; Sobrino-López and Martín-Belloso, 2008), Gram negative bacteria are generally not as sensitive. However, novel variants, such as nisin S29A and S29G, with enhanced activity towards Gram negative food-associated pathogens exist (Field et al., 2012). Nisin A may also be effective against Gram negatives if their outer membrane is destabilized with chelating agents (Stevens et al., 1991). Membrane disruption/permeabilisation is also thought to be the basis for the observation that nisin, when combined with the phenolic compounds carvacrol and thymol which possess membrane permeability properties, exhibit enhanced activity against Gram negative bacteria by permitting nisin to pass through the protective outer membrane (Helander et al., 1998). In fact, there are several studies demonstrating that nisin and essential oil combinations exhibit enhanced inhibitory effects against both Gram positive and Gram negative bacteria (Ettayebi et al., 2000; Olasupo et al., 2003, 2004; Periago and Moezelaar, 2001; Pol and Smid, 1999; Yuste and Fung, 2004). More recently, nisin-containing semi-purified preparations in combination with carvacrol and trans-cinnamaldehyde were established to more effectively inhibit *L. monocytogenes* than either treatment alone (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 $sdH_20$, 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 7000 r.p.m. for 20 min and the supernatant retained. The cell pellet was resuspended in 300 ml of 70% isopropanol 0.1% TFA and magnetically stirred for 3 h at room temperature. Cell debris was removed by centrifugation at 7000 r.p.m. for 20 min 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 160 ml and the sample pH adjusted to

**Table 1**
List of nisin-producing strains and *lux*-tagged bacterial strains used in this study, including relevant characteristics and references.

| Strains/Lux-tagged bacteria | Relevant characteristics or source of strains | Reference |
|---|---|---|
| *Lactococcus lactis* NZ9700 | Wild-type nisin producer | (Kuipers et al., 1993; Kuipers et al., 1998) |
| *Lactococcus lactis* NZ9800 | *L. lactis* NZ9700Δ*nisA* | (Kuipers et al., 1993; Kuipers et al., 1998) |
| *Lactococcus lactis* NZ9800 pCI372*nisA* | Wild-type nisin-producing strain | (Field et al., 2008) |
| *Lactococcus lactis* NZ9800 pCI372*nisA*::M21V | Wild-type producing strain + alteration at position 21 | (Field et al., 2010) |
| *Lactococcus lactis* NZ9800 pCI372*nisA*::S29A | Wild-type nisin-producing strain + alteration at position 29 | (Field et al., 2012) |
| *Cronobacter sakazakii* NCTC 8155::p16S*lux*-$P_{help}$ | Isolated from dried milk powder and transformed with p16S*lux*-$P_{help}$ plasmid | UCC Culture Collection |
| *Escherichia coli* O157:H7 TUV 93-0::p16S*lux*-$P_{help}$ | Derived from strain EDL933 and transformed with p16S*lux*-$P_{help}$ | UCC Culture Collection |

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