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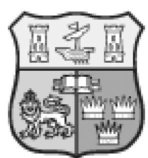

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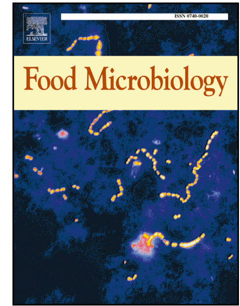

Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control *Cronobacter sakazakii* and *Escherichia coli* O157:H7

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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.

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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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23 **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 µM) 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 µM) 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).

71 Heat treatments and chemical preservatives are commonly used as hurdles
72 to control foodborne pathogens and spoilage bacteria. However, these processes
73 may have undesirable effects, such as altering the nutritional and sensory properties
74 of the food. Furthermore, there has been an increasing consumer demand for
75 additive-free, minimally processed foods, while still maintaining adequate
76 microbiological safety and stability. Therefore, the use of natural antimicrobials as
77 food preservatives has been the focus of ever-increasing attention. Among these
78 natural alternatives are bacteriocins. Bacteriocins are ribosomally synthesised, post-
79 translationally modified peptides that are produced by bacteria and which are active
80 against other bacteria. They can have a narrow range of activity within their own
81 species or a broad spectrum of activity across genera (Cotter et al., 2005). Although
82 there are numerous bacteriocins with food preservation potential, only nisin,
83 produced by *Lactococcus lactis*, is used extensively. Nisin A has been assigned to the
84 lantibiotic class of bacteriocins due to the presence of unusual amino acids that arise
85 due to the post-translational modification of serine and threonine residues
86 ultimately leading to the formation of lanthionine and β-methyllanthionine ring
87 structures, respectively (Bierbaum and Sahl, 2009; Sahl et al., 1995). Nisin A is used
88 in over 50 countries worldwide and has been approved for use by both the EU (E234)
89 and the Food and Drug Administration (FDA) (Delves-Broughton, 1990). Nisin A
90 functions through a unique dual mode of action. It binds to lipid II, an essential
91 precursor to cell wall biosynthesis, while also inserting itself into the bacterial cell
92 membrane. This facilitates pore formation and ultimately leads to the loss of solutes
93 from the bacterial cell resulting in cell death (Wiedemann et al., 2004; Wiedemann
94 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).

119 The aim of this study was to evaluate the antimicrobial activity of nisin A, or the
120 bioengineered nisin derivatives nisin V and S29A, when combined with the
121 essential oils, thymol, carvacrol or trans-cinnamaldehyde, or citric acid against
122 the Gram negative pathogens *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} or *E. coli*
123 O157:H7 TUV 93-0::p16S*lux*-P_{help}.
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132 **2. Materials and Methods**
133
134 *2.1 Bacterial strains and growth conditions*
135
136 The nisin producing strains and *lux*-tagged bacterial strains used in this study
137 are listed in Table 1. *L. lactis* strains were grown in M17 broth (Oxoid) supplemented
138 with 0.5% glucose (GM17) or GM17 agar at 30°C. *E. coli* and *C. sakazakii* cultures
139 were grown in Luria-Bertani (LB) broth or agar at 37°C. When required, antibiotics
140 were used where indicated at the following concentrations: chloramphenicol at 10
141 µg ml^{-1} for *L. lactis* and erythromycin at 500 µg ml^{-1} for *E. coli* and *C. sakazakii*. Stock
142 solutions of thymol (Sigma) were prepared at 50 mg ml^{-1} in 50% ethanol and stored

143 at -20°C. Carvacrol and trans-cinnamaldehyde (Sigma) were diluted from stock
144 (0.976 g ml^{-1} and 1.05 g ml^{-1}, respectively) in 50% ethanol to the desired
145 concentration. Stock solutions of Nisaplin (Sigma) and citric acid (Sigma) were
146 prepared at 100 mg ml^{-1} and 500 mM in sdH_20, respectively, filter sterilised and
147 diluted to the desired concentration. In all experiments, the concentration of ethanol
148 did not exceed 2% (vol/vol).
149
150 *2.2 Nisin purification*
151
152 Purification of wild type nisin A and nisin derivatives were carried out as
153 described previously (Field et al., 2010). Briefly, overnight cultures of nisin producing
154 strains were grown in GM17 broth at 30°C and were subsequently inoculated into
155 two litres of purified TY broth at 1% and incubated overnight at 30°C. The culture
156 was centrifuged at 7,000 r.p.m. for 20 minutes and the supernatant retained. The
157 cell pellet was resuspended in 300ml of 70% isopropanol 0.1% TFA and magnetically
158 stirred for 3 h at room temperature. Cell debris was removed by centrifugation at
159 7,000 r.p.m. for 20 minutes and the supernatant retained. The supernatant was
160 applied to a 60 g Amberlite bead (Sigma) column, which was subsequently washed
161 with 500 ml of 30% ethanol and the inhibitory activity eluted in 500 ml of 70%
162 isopropanol 0.1% trifluoroacetic acid (TFA). The isopropanol was evaporated off
163 using a rotary evaporator (Buchi) to a volume of 160ml and the sample pH adjusted
164 to approximately 4.2. The sample was applied to a 10g (60ml) Varian C-18 Bond Elut
165 Column previously pre-equilibrated with HPLC water and methanol. The column was
166 washed with 120 ml of 30% ethanol and the inhibitory activity eluted in 60 ml of 70%

167 isopropanol 0.1% TFA. Six millilitres of the lantibiotic preparation was concentrated
168 to 1 ml through the removal of the isopropanol by rotary evaporation and applied to
169 a Phenomenex C12 reverse-phase (RP)-HPLC column, previously equilibrated with
170 25% isopropanol 0.1% TFA. The column was then developed in a gradient of 30%
171 isopropanol 0.1% TFA to 60% isopropanol 0.1% TFA from 10 to 45 minutes at a flow
172 rate of 2.1 ml min^{-1}. Fractions containing nisin A and nisin derivative peptides were
173 collected and subjected to Mass Spectrometry with a Shimadzu Biotech MALDI-TOF
174 Mass Spectrometer (AXIMA-CFR plus model).

175

176 *2.3 Mass spectrometry*

177

178 For colony mass spectrometry analysis, bacteria were collected with sterile
179 plastic loops and mixed with 50 ml of 70% isopropanol adjusted to pH2 with HCl. The
180 suspension was vortexed, centrifuged at 14,000 r.p.m. for 2 minutes and the
181 supernatant retained for analysis. Mass spectrometry in all cases was performed
182 with an Axima CFR plus matrix-assisted laser desorption/ionisation time-of-flight
183 (MALDI TOF) mass spectrometer (Shimadzu Biotech, Manchester, UK.) A 0.5 µl
184 aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10mg ml^{-1} in
185 50% acetonitrile-0.1% (v/v) trifluoroacetic acid) was placed onto the target and left
186 for 1-2 minutes before being removed. The residual solution was then air dried and
187 the sample solution (re-suspended lyophilized powder or colony mass spectrometry
188 supernatant) was positioned onto the pre-coated sample spot. Matrix solution (0.5
189 ml) was added to the sample and allowed to air dry. The sample was subsequently
190 analysed in positive-ion reflectron mode.

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193 *2.4 Minimum inhibitory concentration (MIC) assays*

194

195 The MIC of nisin peptides against Gram negative organisms were carried out
196 in triplicate in microtitre plates as previously described (Field et al., 2010). Briefly,
197 96-well microtitre plates were pre-treated with phosphate buffered saline (PBS)
198 containing 1% (w/v) bovine serum albumin (BSA) and subsequently incubated at
199 37°C for 30 minutes. Wells were washed with PBS and allowed to air-dry before the
200 addition of 100 µl double-strength LB broth. Gram negative strains were grown
201 overnight in LB broth at 37°C, subcultured into fresh LB broth and grown to mid-
202 logarithmic phase (OD_{600nm} ~ 0.5). The cells were harvested by centrifugation,
203 washed with 10 mM SPB at pH 7.4 and diluted to 1 $\times 10^5$ cfu ml^{-1} in 10 mM SPB pH
204 7.4. Nisin and nisin derivative purified peptides were resuspended in double-
205 strength LB broth to a stock concentration of 60 µM. Peptides were then adjusted to
206 a starting concentration of 15 µM and 2-fold dilutions of the nisin peptides were
207 carried out in the 96-well plates. Target organisms were added and plates were
208 incubated at room temperature overnight (~16 h). The MIC was taken as the lowest
209 concentration of peptide at which growth was inhibited. The MIC of essential oils
210 against Gram negative strains were carried out as above but with minor variations;
211 BSA treatment of 96-well plates were not required and essential oils were diluted to
212 a starting concentration of 2 mg ml^{-1} for serial dilution of thymol, carvacrol and
213 trans-cinnamaldehyde.

214

215 *2.5 Growth/Kill assays*

216

217 Growth and kill assays were carried out using representative strains as a
218 consequence of the limited amount of pure material available. For growth assays,
219 overnight cultures of target strains were harvested by centrifugation, washed in 10
220 mM SPB pH 7.4 and transferred (1 × 10^7 cfu ml^{-1} in a 1.0 ml volume) into LB broth
221 containing nisin purified peptide alone and in combination with one of the essential
222 oils being investigated. A volume of 200 µl was transferred to a 96-well plate
223 (Genetix) and cell growth was measured spectrophotometrically using a Spectra Max
224 340 spectrophotometer (Molecular Devices, Sunnyvale, California) for 24 h. For kill
225 assays, overnight cultures of target strains were again harvested by centrifugation,
226 washed in 10 mM SPB pH 7.4 and transferred (1 × 10^7 cfu ml^{-1} in a 0.5 ml volume)
227 into LB broth containing nisin purified peptide alone and in combination with one of
228 the essential oils. Samples were incubated for 3 h at room temperature before serial
229 dilution and enumeration on LB agar plates. All experiments were carried out in
230 triplicate.

231

232 *2.6 Infant milk formula trial*

233

234 Commercially available powdered infant formula (PIF) (Aptamil™ First Milk)
235 was prepared according to manufacturer's instructions and brought to room
236 temperature. The pH of the reconstituted PIF was 6.8 as determined with a pH
237 meter. An overnight culture of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} was washed in
238 10 mM SPB pH 7.4, diluted and inoculated into reconstituted PIF at a final

239 concentration of 1×10^5 cfu ml^{-1}. PIF samples were treated with 201.12 µg ml^{-1} (60
240 µM) of nisin A, nisin V or nisin S29A, alone and in combination with carvacrol at a
241 concentration of 300 µg ml^{-1}. Samples with carvacrol alone, *C. sakazakii* NCTC
242 8155::p16S*lux*-P_{help} alone and PIF alone served as controls. Samples were incubated
243 at room temperature and *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} levels were
244 determined through serial dilution and plate count technique on Druggan-Forsythe-
245 Iversen (DFI) agar at 3 h. Where Nisaplin (Sigma), and citric acid (Sigma) were
246 employed, concentrations of 10 mg ml^{-1} and 30 mM were used, respectively.
247 Nisaplin (containing 2.5% nisin) was resuspended in sdH_2O and filter sterilised before
248 use. The addition of citric acid did not significantly alter the pH of PIF. All
249 experiments were carried out in triplicate.

250

251 *2.7 Apple juice trial*

252

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

262 plate count technique on Sorbitol MacConkey agar at 3 h. All experiments were
263 carried out in triplicate.
264
265 *2.8 Statistical analysis*
266
267 CFU data was transformed to $\log_{10}$ prior to analysis using the statistical software
268 package GraphPad Prism 6. All comparisons were based on the mean ± standard
269 deviation. Statistical significance was determined via GraphPad prism t-test. In all
270 cases, a P value less than 0.05 were considered to be statistically significant.
271 Parametric data was analysed using one-way analysis of variance (ANOVA)
272 followed by Tukey's multiple comparisons test. Non-parametric data was analysed
273 using the Kruskal-Wallis one way ANOVA followed by Dunn's multiple comparisons
274 test. Asterisks rating of *, **, *** or **** indicates statistically significant differences
275 between groups (P≤0.05, P≤0.01, P≤0.001, P≤0.0001, respectively).
276
277 **3. Results**
278
279
280 *3.1 Minimum inhibitory concentration assays*
281
282 Nisin V and nisin S29A are previously generated nisin derivatives that exhibit
283 enhanced activity against a number of targets arising from single amino acid
284 alterations (Fig. 1) (Field et al., 2012). These peptides and nisin A were purified and,
285 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 μM and 7.5 μM, respectively) and *C. sakazakii* NCTC
292 8155::p16S*lux*-P_{help} (1.87 μM and 3.75 μM, 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 μM and 7.5 μM, 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 μg 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 μg ml^{-1}. These values are consistent with those previously
307 reported (Hyldgaard et al., 2012).

308

309 *3.2 Growth and kill-curve assays*

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311 Having shown the increased specific activity of the nisin variants against *C.*
312 *sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-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 μM and 15 μM nisin peptides against *C. sakazakii* NCTC
317 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}, respectively, was
318 tested along with varying concentrations (75-200 μg ml^{-1}) of essential oils. When
319 nisin V was used in combination with 100 μg ml^{-1} thymol and 125 μg 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::p16S*lux*-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-cinnamaldhyde (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::p16S*lux*-P_{help}
330 show that combinations of nisin V and thymol (100 μg ml^{-1}) or carvacrol (200 μg 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 carvocrol (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 µg 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 µg ml^{-1} thymol, 300 µg ml^{-1} carvacrol or 350 µg 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

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358 thymol (~2-log reduction), carvacrol (~4-log reduction) and trans-cinnamaldehyde
359 (~1.5-log reduction) against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (Fig. 4). A similar
360 significant level of inactivation of *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} was
361 achieved when thymol (~4.5-log reduction), carvacrol (~3-log reduction) and trans-
362 cinnamaldehyde (~2-log reduction) were used in combination with nisin peptides
363 (Fig. 5). It was also established that the use of bioengineered nisin derivatives in
364 combination with essential oils was more effective than nisin A-essential oil
365 combinations. A significantly greater reduction in *E. coli* O157:H7 TUV 93-0::p16S*lux*-
366 P_{help} was observed when thymol was used in combination with nisin V or nisin S29A,
367 rather than nisin A ($P<0.05$) (Fig. 5A). A similar observation was made when trans-
368 cinnamaldehyde and *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} were utilised, in that a
369 considerable reduction in viable cell numbers was observed when the essential oil
370 was used in conjunction with nisin V (Fig. 4C). Furthermore, an increased bactericidal
371 activity was seen when combinations of nisin V and nisin S29A with carvacrol were
372 used, resulting in a 2-log greater reduction in *C. sakazakii* NCTC 8155::p16S*lux*-P_{help}
373 cell counts than was the case when a nisin A-carvacrol combination was used (Fig.
374 4B).
375
376 *3.4 Model food trials*
377
378 Having shown the enhanced potency of bioengineered nisin variants in
379 combination with carvacrol against both Gram negative pathogens using kill curve
380 assays, this essential oil was selected for further investigation in model food systems.
381 To this end, commercially available powdered infant formula milk and commercially

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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 μM) were added alone or in
385 combination with carvacrol (300 μg 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

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

407 The commercially produced apple juice was filtered and the pH measured.
408 Nisin A, nisin V or nisin S29A (30 µM) was added to the juice alone or in combination
409 with low level of carvacrol (75 µg ml^{-1}) before *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}
410 was introduced at a concentration of 1×10^5 cfu ml^{-1}. Following incubation for 3 h at
411 room temperature, serial dilutions and plate counts on Sorbitol MacConkey agar
412 were carried out to enumerate bacterial cell counts. When nisin A, nisin V, nisin S29A
413 or carvacrol were used alone, an approximate 1-log reduction in *E. coli* O157:H7 TUV
414 93-0::p16S*lux*-P_{help} cell numbers were obtained. The effectiveness of nisin A was
415 significantly improved when used in conjunction with carvacrol, resulting in a 2.5-log
416 reduction in cells counts compared to that of the initial inoculum. Notably, the
417 combinations of nisin V and nisin S29A with carvacrol were even more effective in
418 that a 3-log reduction in bacterial cell counts was attained over the 3 h period (Fig.
419 8). More importantly, these results demonstrate that the enhanced effectiveness of
420 nisin variants observed using laboratory-based assays are retained and can be
421 translated to food systems.

422

423 **4. Discussion**

424

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

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429 sensitivity of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-
430 0::p16S*lux*-P_{help} to nisin. Our investigations also highlight the enhanced potency of
431 nisin when combined with trans-cinnamaldehyde. In the latter case, the mechanism
432 involved may be different as trans-cinnamaldehyde does not disintegrate the outer
433 membrane like carvacrol and thymol, but is believed that at sub-inhibitory
434 concentrations, inhibits the activity of trans-membrane ATPase (Gill and Holley,
435 2006a, b).

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

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

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

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

483 The use of essential oils in foods has been limited due to the high
484 concentrations required to achieve sufficient antimicrobial activity (Hyldgaard et al.,
485 2012). Notably, however, this study shows that relatively low concentrations of
486 carvacrol (approximately 0.0075%) could be used in combination with nisin to inhibit
487 *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} in apple juice. Before commercial
488 application, sensory studies would have to be carried out to determine the
489 organoleptic properties of apple juice with essential oils. Although, upon addition of
490 such a low concentration of carvacrol in this study, no adverse aroma was observed.
491 Recently, sensory evaluations were carried out to assess the consequence of adding
492 75 µl L^{-1} (~63.75 µg ml^{-1}) of lemon essential oil to apple juice. Researchers found that
493 this relatively small concentration of essential oil did not decrease the acceptability
494 of the sample or the organoleptic properties (Espina et al., 2012). Moreover, all of
495 the tested essential oils have GRAS status by both the EU and the FDA (EU, 2012;
496 FDA, Revised 2014a, b) meaning that their addition could be permitted if they were
497 used in the minimum quantities and with good manufacturing processes.

498

499 **5. Conclusion**

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500 The combination of nisin peptides and essential oils could pave the way for
501 new hurdle concepts when it comes to food preservation, in particular towards
502 Gram negative bacteria. Moreover, the combinatory effects could lead to reduced
503 treatment intensity and/or antimicrobial dosage and therefore avoid undesirable
504 sensory and nutritional properties in food. Such combinations could enhance food
505 safety, shelf life and quality while also meeting consumer demands for more natural,
506 preservative-free foods.
507

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509

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699 **Tables**

700

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* NZ9700ΔnisA	(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 p16Slux-P_{help} plasmid	UCC Culture Collection
Escherichia coli O157:H7 TUV 93-0::p16S*lux*-P_{help}	Derived from strain EDL933 and transformed with p16Slux-P_{help}	UCC Culture Collection

701

702 **Table 1**

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703 List of nisin-producing strains and *lux*-tagged bacterial strains used in this study,

704 including relevant characteristics and references.

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Indicator organism	Nisin A µg ml^{-1} (µM)	Nisin V µg ml^{-1} (µM)	Nisin S29A µg ml^{-1} (µM)	Thymol µg ml^{-1}	Carvacrol µg ml^{-1}	Cinnamalde-hyde µg ml^{-1}
C. sakazakii NCTC 8155::p16S*lux*-P_{help}	12.57 (3.75)	12.57 (3.75)	**6.28 (1.875) (P<0.001)**	125	250	250
E. coli O157:H7 TUV 93-0::p16S*lux*-P_{help}	25.14 (7.5)	**12.57 (3.75) (P<0.001)**	**12.57 (3.75) (P<0.001)**	250	250	250

715

716

717 **Table 2**

718 Minimum inhibitory concentration determinations of nisin A, nisin V, nisin S29A and

719 the essential oils thymol, carvacrol and trans-cinnamaldehyde against *C. sakazakii*

720 NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}. Results are

721 expressed as the mean of triplicate assays. Values in bold represent statistical

722 difference between nisin variants and wild type nisin A (Student's t-test: P<0.05).

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736 **Figure legends**
737
738 **Fig. 1.** Structure and mass spectrometry analysis of nisin A and its derivatives. **(A)**
739 Structural composition of nisin A. Blue circles indicate where amino acids were
740 altered resulting in the generation of nisin variants with enhanced activity.
741
742 **Figure 2.** Growth curve analysis of *C. sakazakii* NCTC8155::p16S*lux*-P_{help} with 30μM
743 nisin A (open square), **(A)** nisin V (open diamond), 100μg/ml thymol (open circle) and
744 combinations of nisin A and thymol (closed square), nisin V and thymol (closed
745 diamond) **(B)** nisin S29A (open inverted triangle), 100μg/ml thymol (open circle) and
746 combinations of nisin A and thymol (closed square), nisin S29A and thymol (closed

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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

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771 (open circle) and combinations of nisin A and trans-cinnamaldehyde (closed
772 square)and nisin S29A and trans-cinnamaldehyde (closed inverted triangle).
773
774 **Fig. 4.** Kill effect of nisin A derivatives in combination with essential oils against *C.*
775 *sakazakii* NCTC 8155::p16S*lux*-P_{help}. Kill curve analysis of *C. sakazakii* NCTC
776 8155::p16S*lux*-P_{help} with 60 µM of each peptide both alone and in combination with
777 **(A)** 150 µg ml^{-1} thymol (THY), **(B)** 300 µg ml^{-1} carvacrol (CA) and **(C)** 350 µg ml^{-1} trans-
778 cinnamaldehyde (CN) in LB broth after 3 hours at room temperature.
779
780 **Fig. 5.** Kill effect of nisin A derivatives in combination with essential oils against *E. coli*
781 O157:H7 TUV 93-0::p16S*lux*-P_{help}. Kill curve analysis of *E. coli* O157:H7 TUV 93-
782 0::p16S*lux*-P_{help} with 30 µM of each nisin peptide both alone and in combination with
783 **(A)** 150 µg ml^{-1} thymol (THY), **(B)** 300 µg ml^{-1} carvacrol (CA) and **(C)** 350 µg ml^{-1} trans-
784 cinnamaldehyde (CN) in LB broth after 3 hours at room temperature. ND, not
785 detected.
786
787 **Fig. 6.** Combinations of nisin derivatives, carvacrol and citric acid against *C. sakazakii*
788 NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula. Model food analysis of *C.*
789 *sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula with 60 µM of
790 each nisin peptide both alone and in combination with **(A)** 300 µg ml^{-1} carvacrol (CA),
791 **(B)** 30 mM citric acid (CT) and **(C)** 300 µg ml^{-1} carvacrol and 30 mM citric acid after 3
792 hours at room temperature.
793

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794 **Fig. 7.** Combinations of commercial Nisaplin, carvacrol and citric acid against *C.*
795 *sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula. Model food
796 analysis of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula
797 with 10 mg ml^{-1} of commercial Nisaplin (Sigma) containing 2.5% nisin both alone and
798 in combination with 300 µg ml^{-1} carvacrol (CA) and 30 mM citric acid (CT) after 3
799 hours at room temperature. ND, not detected (detection limit of 10^2 cfu ml^{-1}).
800
801
802 **Fig. 8.** Combinations of nisin A derivatives and carvacrol against *E. coli* O157:H7 TUV
803 93-0::p16S*lux*-P_{help} in apple juice. Model food analysis of *E. coli* O157:H7 TUV 93-
804 0::p16S*lux*-P_{help} in apple juice with 30 µM of each nisin peptide both alone and in
805 combination with 75 µg ml^{-1} carvacrol (CA) after 3 hours at room temperature. ND,
806 not detected (detection limit of 10^2 cfu ml^{-1}).
807
808

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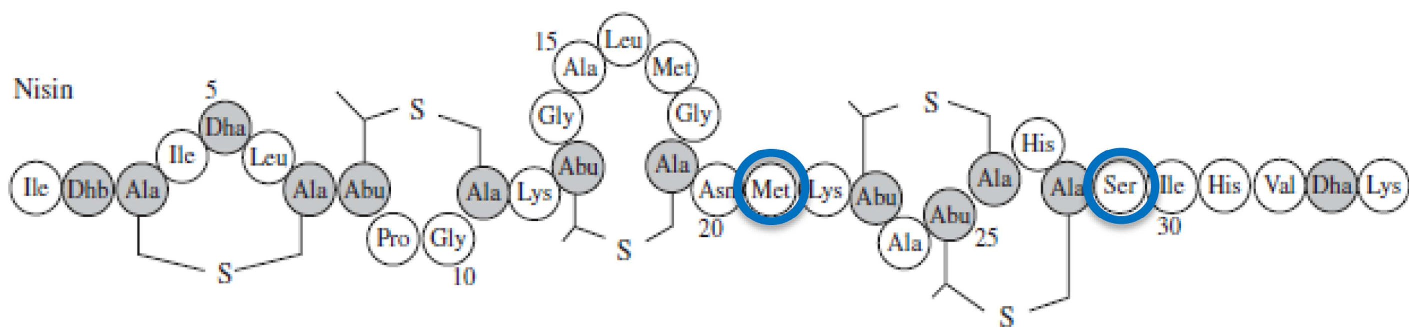

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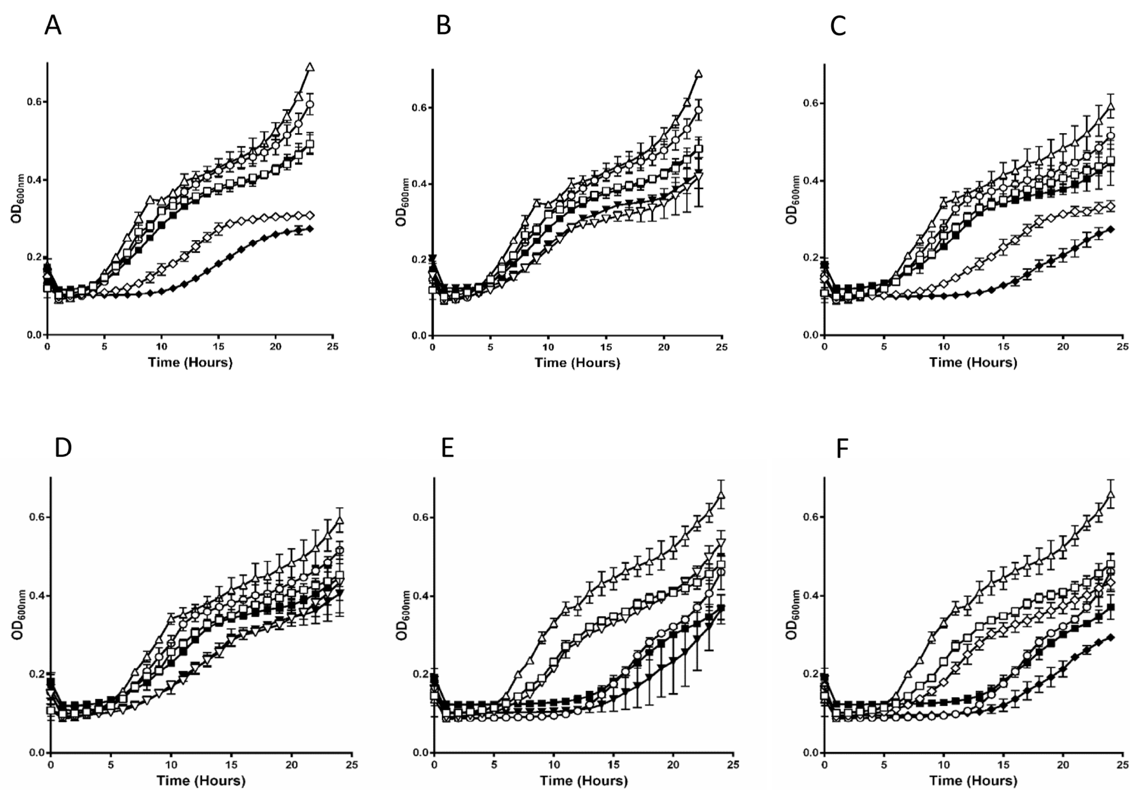

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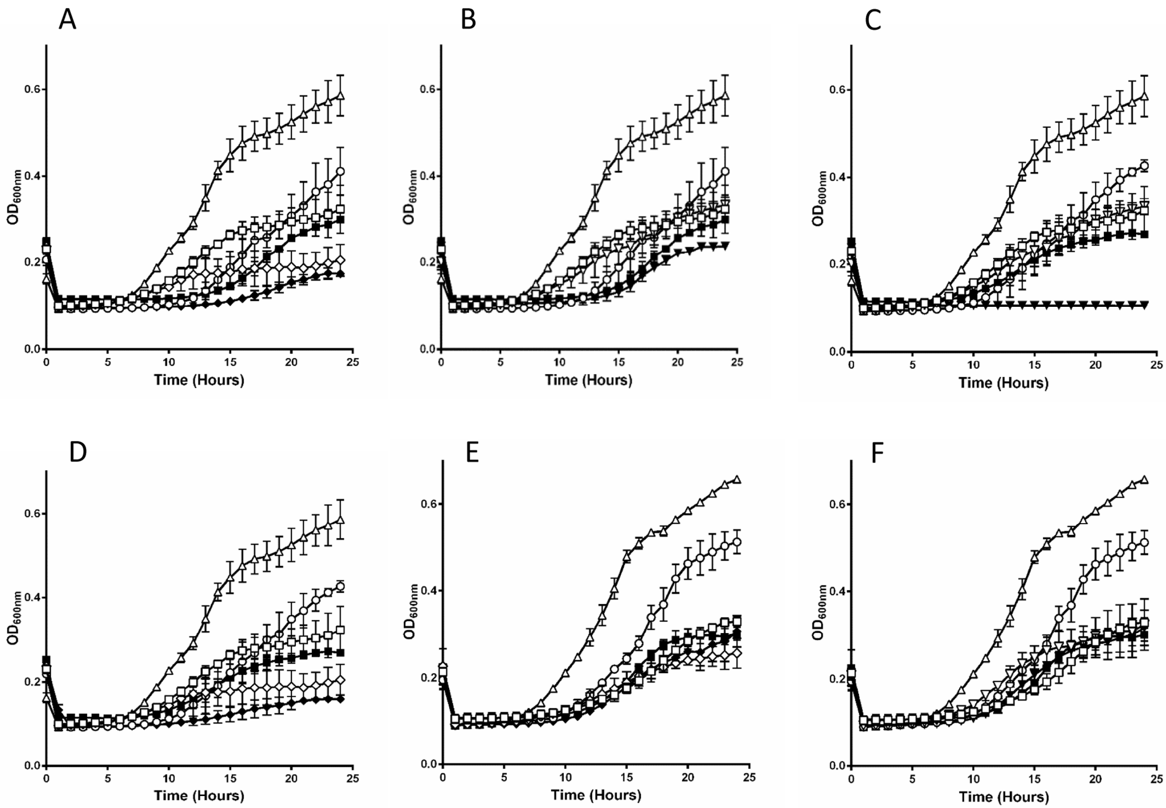

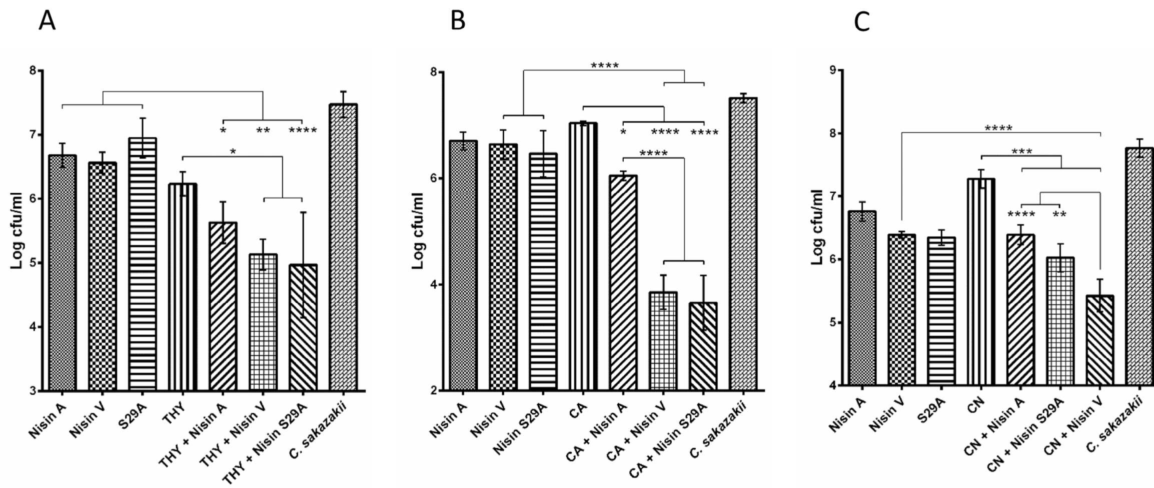

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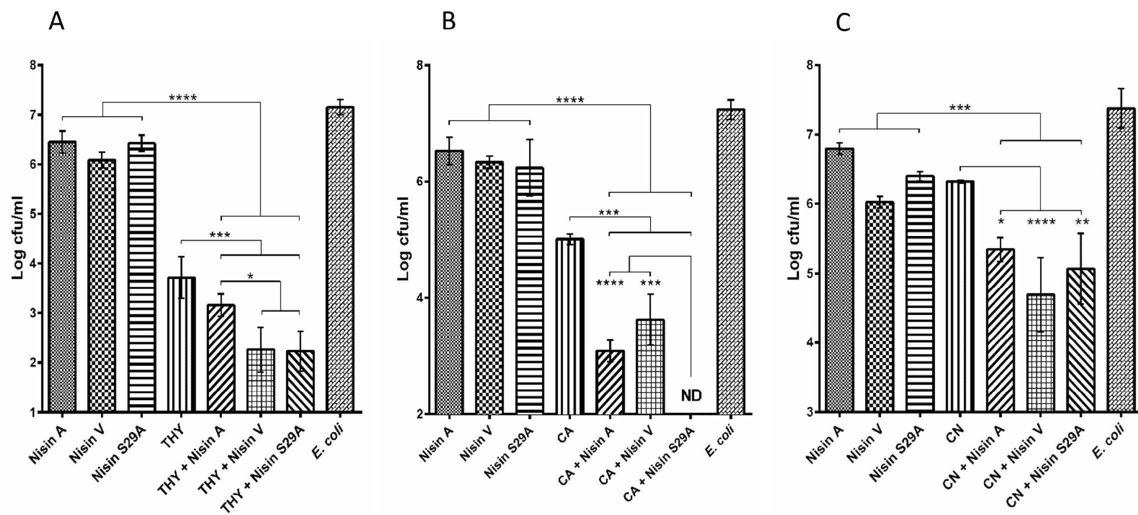

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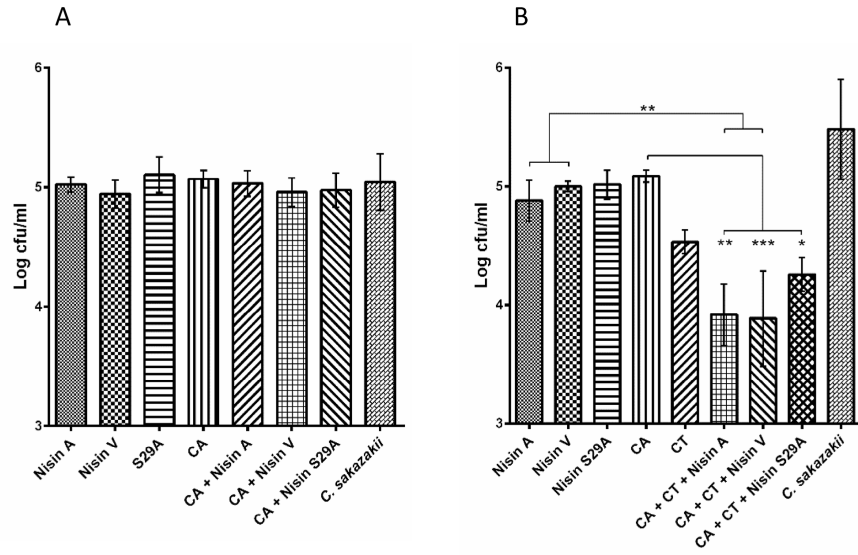

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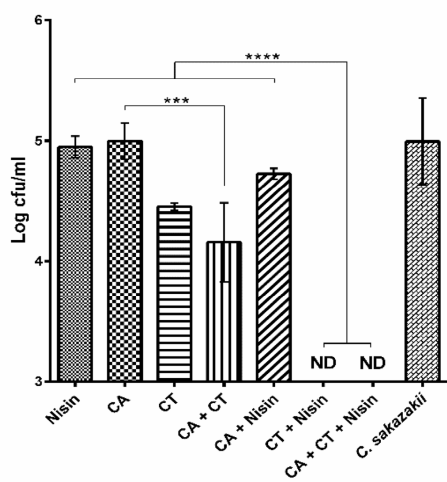

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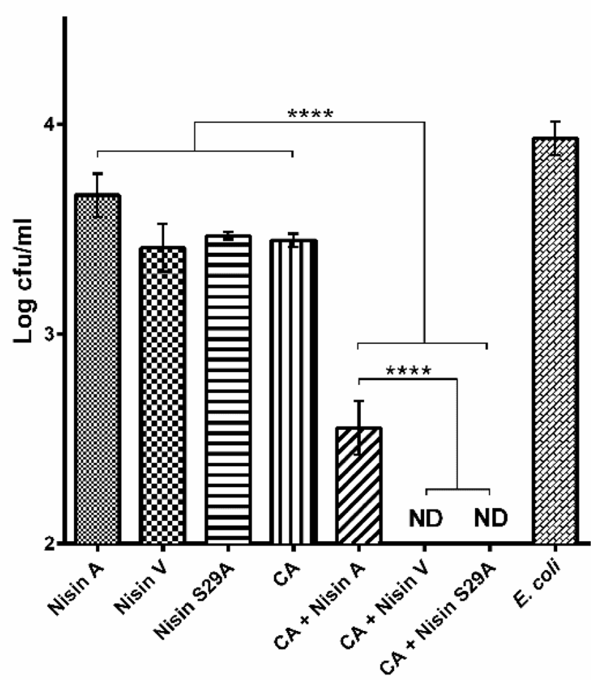

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